THE ASSOCIATION OF ANAEMIA AND PLASMAPHERESIS IN HYPERCHOLESTEROLAEMIC PATIENTS

by

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This is to certify that this thesis is my own work and has not been presented for a degree at any other University

Signed

LUCILLE WOOD

DEDICATED TO

THE MEMORY OF MY MOTHER

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CHAPTER I

OBJECTIVES OF THE THESIS

BACKGROUND

During the 15 years in which plasmapheresis has been performed in the Department of Haematology at the University of Cape Town and Groote Schuur Hospital I have been responsible for the procedures as a result of which a number of abnormalities have been investigated and reported. The problems include disturbances of haemostasis,[1] aluminium or calcium homeostasis[2,3] and benefits in haematologic emergencies were documented.[4,5] A particular and long-standing interest has been in the control of plasma cholesterol levels using exchange procedures and it was in these patients that anaemia was constantly observed. Despite a literature search and discussions at haemapheresis meetings this association seems not to have been studied.

The sub-normal haemoglobin concentrations were shown to result from absolute iron deficiency and this could not be accounted for solely on a dietary basis but rather from additional blood loss. One source was iatrogenic and took the form of frequent specimen collections. Another was intravascular haemolysis that may complicate cell separation.[6] Thus it was postulated that erythrocytes, in these individuals with sub-optimal stores and hypercholesterolaemia, might be unduly susceptible to shear stresses during circulation. This, in itself, would then reduce their life-span and potentially be aggravated by turbulence generated at either site of a surgically created arteriovenous shunt or passage through the

extra-corporeal pathway. When repeated over many years, such low-level breakdown with urinary loss would deplete stores.

STUDY DESIGN

To test this hypothesis a research protocol was approved by the University and Hospital Ethics and Research Committees with participation requiring informed consent.

Firstly, haemoglobin, red cell indices, plasma ferritin and percentage saturation of transferrin were measured to document iron stores. Dietary availability of iron was estimated both quantitatively and qualitatively using a questionnaire. Secondly losses occurring with the plasmaphereses and in urine was accurately measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES): note was particularly taken of additional iron depletion due to blood sampling. Serum lactic dehydrogenase and free plasma haemoglobin levels were used to screen for haemolysis. Concurrent compensation in red cell production was defined by alterations in erythropoietin concentration, shift in the reticulocyte maturation index, glucose-6-phosphate dehydrogenase and pyruvate kinase levels.

Erythrocyte survival was determined directly with standard radioisotope techniques.

The possibility exists that erythrocytes in these patients may be intrinsically less deformable and therefore more fragile than usual. This would cause increased susceptibility to destruction even prior to treatment with exaggeration during therapeutic plasmapheresis. To this end ektacytometry was used to characterise deformability and fragility in the homozygotes as well as normal individuals.

The central issue was early appreciated as being that of iron deficiency anaemia. Accordingly this was the focus of the methods employed and results reported. Nevertheless, in the interests of completeness and because negative iron balance is known to influence haematopoietic parameters such as thrombocytopoiesis, additional observations were made to characterise such associations. These are separated from the main thrust of the study in supplementary chapters for relevant methodology and results.

The data were collated and statistically examined, using appropriate techniques, to see whether the presence of a membrane lesion was primarily attributable to iron loss or possibly with a contribution from hypercholesterolaemia. Irrespective of the causation such a situation might favour acceleration in the physiologic process of extravascular destruction with the additional mechanical trauma of the exchange leading to cellular breakdown in the circulation with further loss of iron from this compartment as well as in the urine.

PERSPECTIVE

The relevance of this investigation is two fold. Firstly, there is the description of a previously unrecognised haematologic lesion in these patients. Secondly, application of this information has direct clinical applicability.

Pathological increases in plasma cholesterol levels together with a variety of inherited abnormalities occur commonly in the Afrikaner population of sub-Saharan Africa and this is explicable on the founder effect.[7-9] In homozygotes standard therapeutic options have limited benefit so that

plasmapheresis has become established as a standard procedure for lipid control.[10,11] It is in this context that the anaemia was first noticed and the hypochromic and microcytic nature correlated with depleted body iron stores. Since the latter state gives rise to symptoms, may further shorten red cell survival and can itself result in paradoxical hyperviscosity there is good reason to prevent its development by oral iron supplementation. Such an intervention can be anticipated to improve quality of life in patients whose survival is critically dependant on long-term plasma exchange.

CHAPTER II

ABSTRACT

INCIDENCE OF ANAEMIA

Many patients undergo single or brief but intensive plasmapheresis and here a number of abnormalities have been recorded including altered concentrations in trace metals and coagulation proteins but anaemia is not a feature. In contrast those on long-term exchange protocols, as for familial homozygous hypercholesterolaemia (FH), were found in a cohort of six to universally drop their haemoglobin levels.

CHARACTERISATION

Hypochromic and microcytic erythrocytes, together with sub-normal ferritin levels and percentage saturation of transferrin, confirmed iron deficiency. To some extent this could be accounted for by a slight reduction in the amount of iron provided by a qualitatively normal diet. An intact absorbing mechanism was documented by ready response to oral supplementation. Haemosiderinuria could not be demonstrated whereas iatrogenic blood loss was known to result from sampling during the aphereses.

HAEMOLYSIS AS A CAUSATIVE MECHANISM

Screening tests in the form of lactic dehydrogenase and free plasma haemoglobin were within the normal range. As an indirect reflection of shortened survival, stimulated erythropoiesis was demonstrated by a shift in the reticulocyte maturation index coupled with a more immature pattern of glucose-6-phosphate dehydrogenase and pyruvate kinase levels. Radiochromium red cell survival was

significantly shortened in cases studied prior to commencing treatment thereby establishing haemolysis as a new finding in these hypercholesterolaemic individuals. Placement of an arteriovenous fistula, followed by the exchanges, led to significant iron loss in discard fluid and urine: this finding necessitated the use of ICP-AES and was correlated with the gradual depletion of body stores. This set of circumstances is explicable on the development of an additional intravascular component to an already reduced mean life-span.

THE RED CELL LESION

Whilst iron deficiency may impair the integrity of red cell membranes it was interesting to recognise that cholesterol, taken-up in proportion to the prevailing plasma concentration, might similarly alter their behaviour.[12,13] Ektacytometry was used to demonstrate reduced deformability and increased fragmentation occurring *in vitro* under conditions of stress calibrated to approximate those prevailing during flow in the microcirculation.

A UNIFYING HYPOTHESIS

Homozygosity for defective expression of the low density lipoprotein receptor results in severe hypercholesterolaemia. A reduced mean red cell life-span, existing prior to commencing plasma exchange, hints at a lipid-associated contribution to the haemolysis particularly since iron nutrition, reflected in serum ferritin levels, is well preserved in comparison to corresponding values in patients on treatment. Interpretation of this association is difficult because one of the cases has had a portacaval shunt and additional affected individuals were not available for study.

Given this caveat it can nevertheless be speculated that the effete red cells are slightly more rapidly removed by the reticuloendothelial system. Such a mechanism is essentially an enhancement of the physiologic pathway whereby extravascular clearance leads to iron-conservation and thus explains the relatively better stores found when first seen. Thus haemoglobin levels could be maintained by erythropoiesis accelerating to keep pace with shortened survival.

However any additional stress, as would occur with the turbulence created by passage across arteriovenous fistulae or during plasma exchange, is likely to be poorly tolerated by these fragile membranes. In this context any degree of intravascular haemolysis, favouring long-term iron loss in the discard fluid and urine, creates an extra need that cannot be met from a diet that is, in the first place, only marginally adequate. As red cells become hypochromic and microcytic they deform even less readily and potentially compound the already reduced survival.

PERSPECTIVE

Apart from documenting this association between plasma exchange and the development of iron deficiency anaemia there is direct relevance for patient management. Thus the simple expedient of providing oral supplementation to overcome loss during chronic plasmapheresis prevents the development of paradoxical hyperviscosity[14] attributable to added rigidity of the iron deficient cells.[15] Such minimal intervention circumvents symptoms due to this type of anaemia and may also prevent disturbances in work output and physical activity.[16]

CHAPTER III

LITERATURE REVIEW

INTRODUCTION

Cardiovascular disease, primarily in the form of atheroma affecting the coronary and cerebral circulations, remains one of the foremost causes of morbidity and mortality on a world-wide basis. There are striking differences between the very high incidence in Westernised societies and much lower levels found in Chinese and rural African Blacks. This discrepancy highlights the importance of predictive factors and, of these, elevation in the plasma cholesterol is of acknowledged significance.[17,18] It is therefore not surprising that vast amounts of time and money have been invested in seeking to accurately identify the role of this essential lipid in pathogenesis and then to design intervention strategies to blunt its impact on both vascular events and survival.[19]

A logical approach starts with epidemiologic studies to locate high-risk populations such as the Afrikaners[20,21] and then to apply appropriate techniques to define causative cellular and molecular biologic mechanisms. These objectives find common ground in genetically determined familial hypercholesterolaemia (FH) with prevalences of homozygotes and heterozygotes, at least in Transvaal Afrikaners, calculated to be one in thirty thousand and one in one hundred respectively.[22] Even here there exists a clinico-pathological spectrum within the homozygotes and, as anticipated, lesser severity where there is a heterozygous pattern of transmission.[22] This experiment of nature, so well illustrated

in our local population yields, regrettably, individuals who are refractory to dietary manipulation and drug therapy but can be controlled by serial plasma exchange.[10] When this modality is employed over extended periods of time, it is clearly beneficial whether apheresis or affinity-columns are used. Thus reduction in cholesterol level occurs promptly and is sustained while clinical outcome over the longer term is improved.[11]

Immediately following the procedure alterations in plasma protein and other components are relatively well characterised but, of apparently unappreciated significance, are more subtle and potentially ominous consequences that only emerge when follow-up is continued. One of these encountered in plateletapheresis is quantitative alterations in T-lymphocyte subsets.[23] More striking is the previously unreported but gradual development of hypochromic and microcytic anaemia which cannot be accounted for by an inadequate diet. That the problem does not lie with absorption is proven by prompt response to oral iron supplementation. Since haematologic values are normal at presentation a novel concept is that reduction in red cell mass is the end result of protracted periods of additional blood loss where one source was specimen collection and another was intravascular haemolysis that may complicate cell separation.[6] Thus it can reasonably be speculated that erythrocytes in the homozygous state are rendered abnormal by the high lipid environment in which they circulate. Under the latter circumstances they undergo accelerated physiologic removal by the reticuloendothelial system which is an iron-conserving extravascular pathway.

Compensatory mechanisms stimulate erythropoiesis and a younger population is released in numbers adequate to maintain normal blood counts, at least when viewed superficially.

However, when such fragile cells undergo further stress, at points of turbulence created by arteriovenous connections established to maintain ready vascular access, or when compounded by passage through the long extracorporeal plastic circuit in the separators, two events take place. Firstly, there is acceleration in extravascular breakdown and secondly, intravascular haemolysis develops. The latter culminates in iron being lost with the discard plasma as well as urine and, if continued long enough, ultimately contributes to depletion of stores and falling haemoglobin. Apart from the purely scientific interest of testing this hypothesis there is a pragmatic consideration in needing to recognise that the evolution of such a conditioned nutritional deficiency brings with it new symptoms. These side effects can be averted by the simple expedient of providing oral iron supplementation.

Other haematologic complications of aphereses, that may predispose these individuals to varying degrees of morbidity, are all rare. They include impaired phagocytic function, potential disturbances in humoral or cell-mediated immunity[24] or where platelet activation may follow exposure to foreign surfaces and create a risk for acute thrombotic events.[25]

This literature review has therefore been undertaken to place in perspective the impact of long-term plasma exchange required to control the lipid abnormalities with particular emphasis on the pathophysiology of the anaemia and, where

appropriate, to document other haematopoietic changes that may occur.

CHOLESTEROL PHYSIOLOGY

Source, Synthesis and Function

This amphipathic lipid is water insoluble forming, as it does, an integral component of many body cells where it participates either free or covalently bound to fatty acids to yield an ester suitable for intracellular storage. The diet contributes about half of the body pool being derived from meat, liver and egg yolk with the remainder endogenously synthesised by the liver, and to a lesser extent, the gastrointestinal tract and skin. In these pathways the rate of formation is limited by 3-hydroxy-3-methyl glutaryl coenzyme A reductase or HMG-CoA reductase.[26] Understandably the latter becomes the target for modern therapeutic approaches used singly[27] or in combination.[28,29]

Cholesterol is the precursor of adrenocortical and sex hormones as well as bile acids with the lipid itself appearing in gall stones: earlier in the pathway squalene can give rise to vitamin D rather than this coming from cholesterol itself. Normal cell function is critically dependant upon plasma concentrations and when this drops below 150 mg/dl, which is equivalent to 4 mmol/L, may be associated with increased mortality from non-coronary causes[30] but this may be related to the drugs rather than the reduced serum level.[31] It is noteworthy however that these concentrations are seldom, if ever, encountered in homozygous familial hypercholesterolaemia.

Transport and Excretion

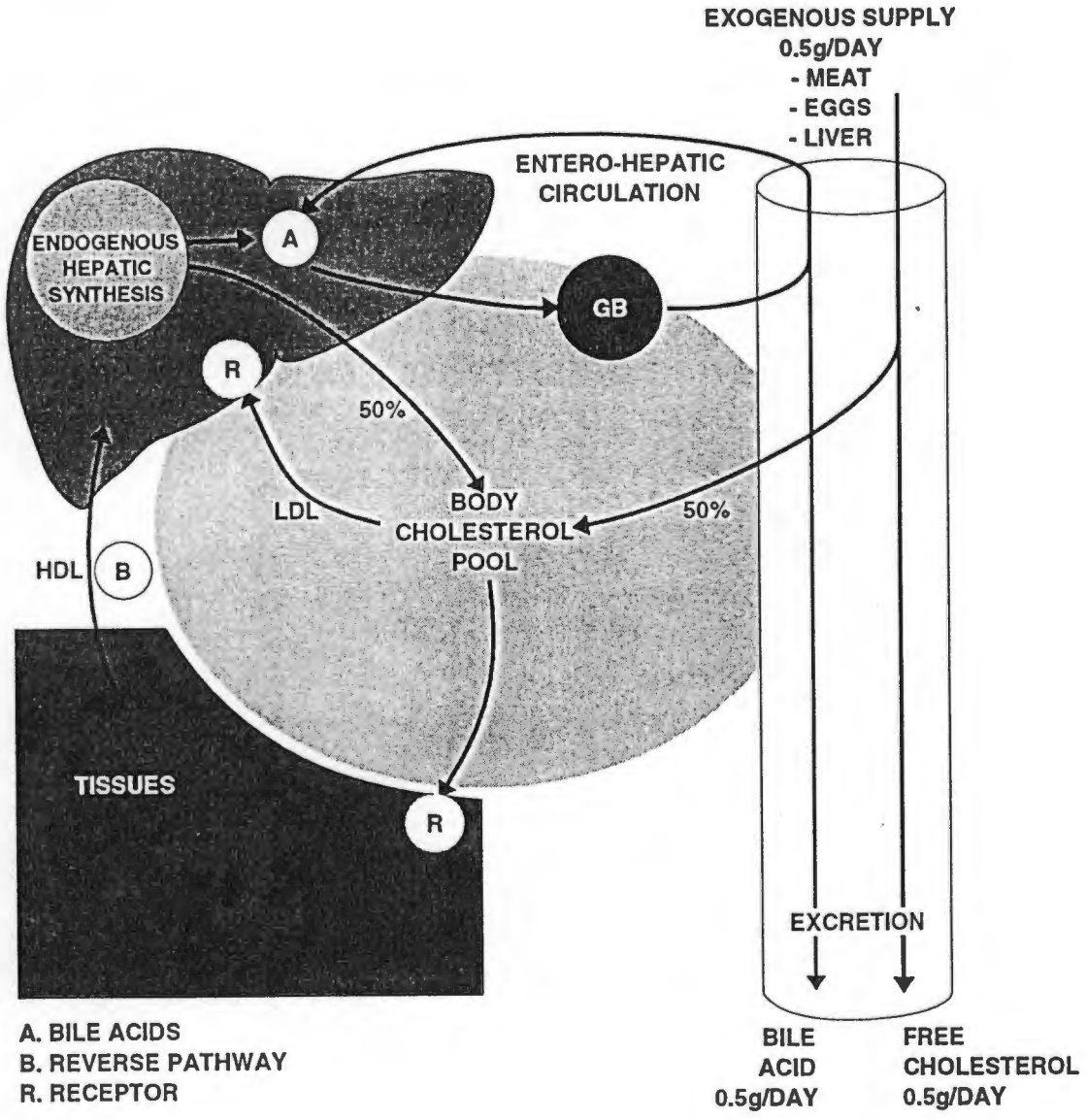
Poor solubility in biological fluids is compensated for by transport within lipoproteins. These comprise apolipoproteins that non-covalently associate with free or esterified cholesterol. When complexed in this way movement is facilitated from the gastrointestinal tract following absorption, as well as from sites of synthesis mainly in the liver to points of utilization. Additionally the high density fraction moves free cholesterol from tissues to the liver, in what is termed the reverse transport pathway, for subsequent conversion to bile salts. In this form approximately half is lost in the faeces as bile acids or their salts and the remainder excreted as neutral steroids (Figure I). A portion of the free cholesterol in the bile is reabsorbed in an enterohepatic circulation. Additionally some of the cholesterol that serves as a precursor for faecal steroids is derived from the intestinal mucosa:[26] a very minor pathway.

PATHOPHYSIOLOGY OF HYPERCHOLESTEROLAEMIA

Elevation in cholesterol is a marker for coronary and, less frequently, cerebral vascular disease with the most serious effects evident in those with FH.[32,33] Within this group the plasma level was noted to vary widely and it remained for Brown and Goldstein[33] as well as others[34] to demonstrate that this partly reflected different mutations in the low-density lipoprotein (LDL) receptor. The latter is a single-chain glycoprotein that contains 839 aminoacids when mature and is encoded for by a gene on the short arm of chromosome 19. Any structural disturbances in the receptor,

FIGURE I

CHOLESTEROL METABOLISM



Plasma cholesterol is derived from the diet in an exogenous pathway and endogenously synthesised by the liver. Its clearance, particularly of the low density lipoprotein fraction, is predominantly mediated by the cognate receptor largely on hepatocytes.

reflecting an inherited defect in its gene, has the capacity to disrupt normal control of cholesterol metabolism.[35]

Genetics

Mutations of the normal LDL receptor gene influence its biophysiology with sub-division between three and five major classes (Table 1).[35-37] This is an autosomal dominant condition and thus the heterozygous form is much more common in the general population. Our interest centres on South African studies where a founder effect is evident in geographically isolated ancestral populations,[9] notably the Afrikaners. Furthermore plasma cholesterol levels in the heterozygotes are generally between 7.5 and 12 mmol/L whereas, in homozygotes, this is typically in the range of 15-25 mmol/L.

In homozygous FH genetic defects that impair rather than abolish receptor function tend to produce lower plasma concentrations of cholesterol. Such patients are more responsive to treatment which consequently results in less severe degrees of cardiovascular disease. This point has recently been reviewed in detail.[21,35]

Since some individuals having the same receptor defect show systematic differences in LDL cholesterol concentration the existence of modulating genes was postulated and confirmed. In both a Puerto Rican and Utah pedigree modifiers having adverse effects on the phenotype have been documented.[38]

Classification

The lipoproteins may be classified according to composition, size and density (Table 2).

TABLE 1

CLASSIFICATION OF LDL RECEPTOR MUTATIONS

CLASS	
I	Failure to produce any detectable receptors.
II	Receptors failed to mature normally.
III	Receptor binding to lipoproteins is poor.
IV	Lack of receptor internalisation.
V	Failure of receptors to recycle.

One hundred and fifty different mutations have so far been described and these code for membrane defects that variably affect the efficiency with which lipoproteins are cleared from plasma.[37]

TABLE 2

LIPOPROTEIN CHARACTERISTICS

Lipoprotein	Chylomicrons	VLDL	IDL	LDL	HDL
Diameter(A)	800-5,000	300-800	250-350	180-280	50-120
Density (g/ml)	<0.94	0.94 -1.006	1.006 -1.019	1.019-1.063	1.063 -1.210
Electrophoretic Mobility	Remains at origin	Pre-beta	Slow pre-beta	Beta	Alpha
Major Lipids	Dietary triglycerides	Endogenous triglycerides	Cholesteryl ester, triglycerides	Cholesteryl ester	Cholesteryl ester
Apolipoproteins	A-I,A-II,A-IV,B-48, C-I,C-II,C-III,E	B-100,C-I,C-II, C-III,E	B-100,C-III,E	B-100	A-I,A-II,C-I,C-II, C-III,D,E

The lipoproteins are classified into their major groups based upon size, reflected in diameter, density, electrophoretic mobility as well as lipid and protein content. The apolipoproteins act as co-factors for enzymes or as ligands for receptors and in this way are centrally involved in the metabolism of lipoproteins.

Firstly the apolipoproteins are currently allocated to one of five groups designated A to E. The apo-B 100 functions as the ligand or attachment site for the low density lipoprotein (LDL) receptor.

These molecules, together with their major lipids, give rise to the lipoprotein complex. These fall into five sub-types or classes designated chylomicrons, very low density lipoproteins or VLDL, intermediate, LDL and high density lipoprotein or HDL. While interactions between them are important most current interest centres on the low-density fraction which, through apolipoprotein B-100, is recognised by its cognate receptor expressed on cell surfaces including, particularly, the hepatocyte.

THE CLINICAL SPECTRUM

Heterozygotes

Such individuals have a prevalence that varies worldwide from about one in five hundred to one in one hundred among Afrikaners and a plasma cholesterol that is typically between 7.5 and 12.0 mmol/L at birth with LDL two to three times above the mean normal value and HDL less impressively raised. This biochemical finding may be the only evidence of disease during the first decade of life. From the age of ten years corneal arcus, thickened tendons and xanthomas appear: in the third decade the latter features are universally found and presage the onset of symptomatic coronary artery disease with early myocardial infarction.[33]

Homozygotes

This inherited metabolic disorder has a reported frequency of about one in a million[33] but one in thirty

thousand in Transvaal Afrikaners.[22] Biochemically there is hypercholesterolaemia greater than 15 mmol/L present at birth and persisting throughout life with variations being accounted for by the type of mutation present. Those with mean plasma levels of 16.1 mmol/L have the exon three mutation as opposed to the greater value of 26.7 mmol/L when the 10kb deletion is present.[34] These figures translate into a higher death rate for the latter group. LDL is typically raised six times above physiologic levels with HDL slightly lower and triglycerides normal or minimally increased.

Clinically there are striking plaques or lemon-orange coloured xanthomas and tendon thickening both of which are evident shortly after birth and have inevitably developed before the age of four. Corneal arcus does not unusually manifest in the first decade. The striking feature is rapidly progressive atherosclerosis with childhood onset of angina pectoris, classical electrocardiographic changes of ischaemic heart muscle and obstructive atheromatous lesions evident on angiocardiography. Myocardial infarction is frequent with death often within the first 30 years of life. Unexplained are episodes of polyarthritis and tenosynovitis.[33] In some instances the features are those of aortic ejection systolic murmurs due to supravalvular stenosis.[22]

Diagnosis is currently best confirmed with DNA-based studies and, if this is conducted as part of an ante-natal screening programme, offers parents the option of considering termination. However it should be noted that this would be contentious in a disorder where effective treatment is

available and some cases have survived for long periods of time without major intervention.

Certain well described although unusual haematologic abnormalities occur in this setting. Importantly red cell damage seems not to have been reported and most notably anaemia is not a recognised association. Conversely hypercholestrolaemia affects platelet function and their survival both experimentally as well as in human disease.[39] Also relatively poorly characterised are changes in leucocytes from such patients which, nevertheless, are abnormal morphologically and when examined with flow cytometry.[40] Intravascular coagulation may be disturbed in these individuals with alterations in antithrombin III levels and fibrin or fibrinogen degradation products. When taken in conjunction with platelet lesions diverse rather than any uniform effect emerges.[41]

TREATMENT

Heterozygotes

Diet was previously advocated to limit total cholesterol to 300 milligrams a day for adults and half this for children with decrease in unsaturated fat intake and saturated products increased. However it is noteworthy that most clinicians would favour even more strict control than these older guidelines. Weight reduction is beneficial in those who are above the ideal body mass and regular exercise is helpful in lowering triglyceride and LDL level whilst elevating the high density lipoproteins. Dietary control is, however, seldom sufficient and invariably pharmacologic intervention is required.

Drugs are most effective when combined with reduced lipid intake.

Bile acid sequestrants, such as cholestyramine, are non-absorbable resins that sequester cholesterol retaining it within the intestinal tract leading to significant loss in the faeces: consequently the amount available for lipoprotein production by the liver falls markedly.[42,43] Furthermore, by increasing cell surface receptors for LDL, it is possible to bring about a 20 to 30 percent reduction of this lipoprotein whereas those for high density molecules may rise 5 to 10 percent.[43] Since these therapeutic agents are not absorbed systemic toxicity does not occur: they are however unpalatable and lead to poor compliance.

Nicotinic acid or niacin is a water soluble vitamin that reduces the flux of non-esterified fatty acids to the liver with a consequent reduction in lipoprotein secretion.[43] It can lower cholesterol by 20 to 30 percent, triglycerides between 20 and 60 percent and may lead to a rise in high density lipoprotein by 20 to 30 percent. Unfortunately the dose required is associated with significant side effects.

Probucol has an uncertain action but appears to enhance non-receptor mediated LDL clearance. Although plasma levels drop 5 to 15 percent it, at the same time, decreases HDL by up to 25 percent and is therefore not widely used at the present time.[43]

Fibrates such as bezafibrate, fenofibrate, gemfibrozil act by accelerating the activity of lipoprotein lipase so that triglycerides fall some 30 to 60 percent, serum cholesterol 5 to 20 percent whereas HDL rises 10 to 15

percent. These changes are well tolerated and associated with few if any symptoms.[43]

Three-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) reductase inhibitors such as simvastatin, pravastatin and lovastatin[11,27,44] inhibit the enzyme which is rate-limiting for cholesterol synthesis and thereby deprives intracellular metabolic processes of this essential precursor. In consequence there is forced up-regulation of the LDL receptor in order to compensate for its limited availability with faster clearance of LDL and consequential decrease in serum concentrations. These are promising agents and have the most dramatic effect of all drugs in decreasing LDL cholesterol in the heterozygous FH and persons with normal LDL receptor status. There are only minor symptoms with tolerance usually being good. Although low cholesterol levels are associated with behavioural changes these are seldom found in FH and, indeed, there is a question as to whether adverse effects may not be related to the drug itself.[45]

Partial ileal bypass produces some therapeutic impact in heterozygotes and is approximately equivalent to that achieved with bile sequestrants such as cholestyramine.[46]

Plasmapheresis has been used but evidence that it is clinically useful is less convincing than in homozygotes. It seems to offer no real advantage over conventional therapy.

The preferred treatment is by strict attention to diet combined with one or other of the drugs and, of these, HMG CoA reductase inhibitors are most effective in reducing both serum cholesterol and particularly the LDL fraction.

Homozygotes

Diet is essentially similar to that employed in the heterozygous state but here it is relatively ineffectual although nevertheless useful.

Portacaval anastomosis has been created in a number of these individuals. However, the outcome of this surgical procedure is variable but, in a percentage of cases,[47] may reduce cholesterol by up to fifty percent: a further advantage is impaired synthesis of bile salts that bring about drop in LDL concentration.

Drugs are effective but the extent to which this occurs is determined by the presence or absence of LDL receptor binding activity. If mutations have abolished this function all forms of therapy are valueless. In contrast retention of even varying degrees of ligand-binding can result in significant lowering of the cholesterol level by diet in combination with one or other of the same agents employed in managing the heterozygote. Generally speaking lipid-modifying agents are not, however, routinely employed in homozygous FH.

Plasmapheresis is successful and a widely employed therapeutic option for direct removal of the offending LDL: this includes the variation in which affinity columns are used to selectively deplete just the particular fraction. Either way these aphereses procedures are well tolerated but require lifelong performance with intervals of one to two weeks. Cholesterol can be reduced by 50 percent.[10,11,48,49]

Liver transplantation is the most direct way of overcoming the genetically determined defect in the homozygotes. As more than three quarters of the total receptor

population in the body are located on this organ, there is rationale for such an aggressive approach. These individuals then become responsive to drug therapy that will stimulate activity of these LDL-binding structures.[50]

Gene therapy is promising and animal studies have demonstrated that sustained and safe lowering of LDL can be achieved: this offers an attractive therapeutic option for the future.[51]

APHERESIS TECHNOLOGY

Impact on Cholesterol

In general terms this is adjunctive to optimum combination of diet and receptor stimulating drug programmes. These procedures are used relatively infrequently in heterozygotes but are the cornerstone of treating patients who are homozygous particularly where the mutation is such that alternative and less invasive approaches are ineffective. Plasma cholesterol levels can be reduced by half provided these are carried out on a long-term basis.[52,53]

Centrifugal Separators

Introduced in the early 1950's for collecting blood cells, these instruments have undergone extensive technological improvement in the last four or more decades.[54] The modern versions are efficient and safe in that the older polycarbonate bowls have been replaced by disposable plastic lines. The principle is fractionation of whole blood in a centrifugal field based on particle density. The process may be either continuous or intermittent[55] but in either of these two cases there is non-selective removal of the plasma together with all that it contains including both low and high

density lipoproteins. Apart from safety the latest generations are fully computerised, relatively inexpensive to operate and have a high degree of patient acceptability.

Filtration Techniques

These depend upon the isolation and retention of cells either by passage through flat cellophane sheets or hollow nylon fibres.[54] The process is governed by size of the molecule and efficiency determined by a number of variables that include packed cell volume, viscosity, flow rate, number and dimensions of the channels in the module.[56] The extracorporeal blood volume is usually small and an almost totally cell-free discard readily achievable.

Immunoadsorption Columns

Plasma, which can be prepared by any of the different methods, is perfused through an affinity column which contains a dextran sulphate-cellulose gel base rich in binding sites for low density lipoprotein cholesterol.[49,57] Alternatively an anti-human LDL antibody, directed against apolipoprotein B100, is coupled to a sepharose matrix.[58] In both cases the offending fraction can be specifically depleted while other components are returned to the patient thus obviating the need for any form of fluid replacement.[49]

The HELP or Heparin Extracorporeal LDL Precipitation System

Flocculation of lipids with heparin can be achieved at acidic pH. These large insoluble complexes are then simply removed by filtration:[59] it should be appreciated that there is an associated and often marked reduction in fibrinogen.[59,60]

Immediate Complications

Vascular access is critical to ensure the high blood flow that is necessary for efficient procedures. Frequently antecubital vessels are inadequate and, for this reason, femoral, jugular or subclavian veins may need to be cannulated or even arterio-venous fistulae or shunts created.[61] In the latter situation hazards include loss of a distal arterial segment, stroke or even gangrene. Other significant problems include haemorrhage, thrombosis and infections.[62]

Replacement fluid needs to be carefully constituted. Use of simple electrolyte solutions may result in hypo-proteinaemia, hypovolaemia and drop in blood pressure.[63] This can be circumvented by addition of albumin or group-specific fresh frozen plasma. However the latter should not be used on its own in chronic programmes particularly for hypercholesterolaemia because it does not, as efficiently, reduce the lipoprotein levels. Additionally this material may be associated with allergic reactions including urticaria and pruritus. Brisk haemolysis can occur unless group-specific ABO serum is used to circumvent the possibility of a high titre of naturally occurring IgM antibody destroying the recipients red cells.[62]

Albumin can cause unpredictable pyrexia with aching joints and a feeling of fatigue: neither of these are classical pyrogenic events and despite considerable investigation their mechanism is unexplained.[64] Conversely, allergic reactions of variable severity may occur when individual units contain small amounts of antigen to which the recipient has previously been sensitised and these may vary

from such things as food proteins to medication, for example, sulfonamides or penicillin.

Circulatory effects generally reflect failure to accurately balance extracorporeal loss of fluid although most modern instruments have an electronic circuit with alarms to indicate the development of such disequilibrium. Symptoms of hypovolaemia are often rather non-specific with malaise, headaches, tiredness or cramps but late signs include abdominal pain, vomiting and collapse: these tend to be more pronounced with increasing age. At the other extreme is cardiovascular overload that may cause pulmonary oedema particularly in the elderly. Meticulous attention to detail and maintenance of isovolaemic exchanges should, certainly in experienced hands, be sufficient to avoid such occurrences.[62]

Heparin anticoagulation is an effective approach but it is important that overdosage not occur since this can lead to haemorrhage. Conversely, inadequate quantities predispose to thrombi forming in the circuit.[62] This is associated with reinfusion of partially clotted material that has procoagulant activity and may result in disseminated intravascular coagulation or cell breakdown in the separator. In patients undergoing column-based procedures that involve dextran sulphate, hypocoagulability is sometimes found as factors V, VIII and X are adsorbed and then lost.[65]

Calcium chelation and citrate toxicity have both been well characterised.[66] The hypocalcaemia required to prevent blood clotting may be associated with neuromuscular hyperactivity, sensory disturbances, circumoral parasthesiae, nausea and vomiting: shaking chills may arise and progress to

tetany while cardiac arrhythmias can also occur. All of these emphasise the importance of careful non-invasive monitoring during the entire run.[62,66] Most of the side effects are preventable by prophylactic supplementation with oral calcium salts or titrated doses of either the chloride or gluconate given intravenously during the course of the exchange. Citrate toxicity will be aggravated when the replacement fluids contain large amounts of plasma contaminated with this anion or in the presence of renal failure where its metabolism is impaired.

Effect on medication is markedly influenced by these procedures. Thus many low molecular weight substances are rapidly removed and accordingly schedules need to be adjusted after completion of the apheresis so that therapeutic levels are reinstituted. Examples include anti-epileptic agents or cholinesterase inhibitors.[67] This phenomenon is particularly troublesome with drugs that are protein-bound since such complexes are efficiently cleared from the circulation.

Haemolysis may arise for a number of reasons. For example hypotonic fluids may bring about severe degrees of red cell breakdown.[68] In addition blood exposed to turbulence during mechanical separation develops subliminal alterations in erythrocyte membranes which can lead to accelerated removal probably by the reticuloendothelial system. This is a bigger problem with discontinuous flow instruments and, whilst infrequent, it is mandatory that the operator continuously checks the plasma for colour change and randomly measure free haemoglobin spectrophotometrically.[62]

Air embolism is a hazard but should not occur with meticulous attention to technical details. When this takes place and it is of small volume, symptoms and signs are transient. Occasionally severe and irreversible cerebral damage or cardiac arrest arise when the connectors become detached or the tubing is faulty so that air obstructs small but vitally placed arteries.[62] Complaints include chest pain, shortness of breath, extreme anxiety, a drop in blood pressure with sweating and syncope. Alert staff will detect malfunction since modern equipment has alarms and other suitable warning devices. Nevertheless it is impossible to over emphasise the importance of constant supervision.

Delayed Effects

Haemorrhage is relatively unusual and takes two forms. With some of the older instruments loss of red cells in the discard tubing was sufficient to deplete iron stores. Interestingly a similar situation of iatrogenic phlebotomy can occur with frequent blood sampling. Alternatively blood loss may develop acutely. Rarely, only once in my experience over two decades with many thousands of procedures, has a patient left the unit and developed a major bleed from the femoral site. This was probably mechanical and responded to local pressure: the clotting factors on assay did not significantly differ from the reduced values normally found after plasma exchange.[69] A contributing factor can be loss of platelets which, with properly scheduled intervals, do not reach levels usually associated with bleeding. Furthermore, poorly controlled use of antithrombotic agents producing

hypocoagulable states, has not been described as a cause for this complication.

Thrombosis may be precipitated by removal of naturally occurring anticoagulants such as antithrombin III.[70] Although apparently not studied it would be reasonable to anticipate that concurrent loss of the proteins C and S would occur. If this were so, one consequence, theoretically at least, might be predisposition to hypercoagulability.

Bacterial infections seldom arise despite some decrease in immunoglobulins although, where patients additionally receive immunosuppressive therapy, they become susceptible to this possibility.[61,62,71]

Viral infections are also a risk particularly where plasma is used and here rational concerns extend to the retroviruses most notably that causing the acquired immunodeficiency disease. All products need to be drawn from donor panels that have been carefully screened and recent evidence has shown that this care needs to extend to freedom from hepatitis C.[72]

Protein depletion results only when serial exchanges are carried-out and the replacement does not contain albumin. This may lead to subtle development of hypoproteinaemia, loss of oncotic intravascular pressure and dependant oedema.[61,62,63]

Chills may be troublesome if the room temperature is too low. However they are also found when the individual is unable to change position frequently or in association with the infusion of cold exchange fluid.[62] When immunoabsorption columns containing sheep antibodies are used there may, in addition, be fever and flushing.[73]

Morbidity and mortality are significant although infrequent occurrences. Fifty deaths have been reported of which 30 were due to either acute cardiac arrhythmia or refractory pulmonary oedema.[61] The complication is generally reported as being in the region of three per 10,000 procedures.[61]

Haematologic Consequences

Erythrocytes have attracted scant attention although iron-responsive anaemia has been observed but never investigated.[49,74] Here, rather than examining the mechanism for depletion of stores, simple replacement has been employed for symptomatic control. However, alternative possibilities seem not to have been considered. For example could repeated blood sampling deplete stores? Is it possible that shortened red cell survival may exist as a consequence of the hypercholesterolaemia itself? If so could the latter be aggravated by turbulence during the extracorporeal circulation? This outcome is hinted at by previously reported acute intravascular haemolysis albeit that the patient was receiving cyclophosphamide.[6]

Leucocytes of both major categories become dysfunctional. Although neutrophils are affected this seldom translates into clinical important infections in our experience. Conversely, lymphocytes are quantitatively and qualitatively altered in the closely allied technique of plateletpheresis and here significant and long-lasting alterations in function have been documented.[23,75] By the same token monocytes are known to be activated, perhaps by contact with foreign surfaces such as

the plastic, and then release cytokines with a variety of different effects.[76]

Platelets often drop by 15 to 50 percent depending upon the instrument used.[1] Of note is the observation[69] that thrombocytopenia universally develops acutely but seldom much below the lower limit of normal. Even if aphereses are carried-out serially, and allowing for the direct loss into the discard fluid, recovery is prompt and usual. It has furthermore been shown, using flow-cytometry, that platelet activation occurs in the hypercholesterolaemic patient and, of note, is the fact that these procedures reverse this defect.[77]

The coagulation proteins influence prothrombin and partial thromboplastin times with both increasing modestly although return to baseline is usual within 24 hours.[1,69,78] Other data showed that they would only be prolonged if the fibrinogen level fell to approximately 500mg/L.[69,71] Interestingly clotting factor concentrations are depressed in direct proportion to the volume of fluid exchanged but may be reduced up to 50 percent of the pre-treatment values and often remain down for up to 72 hours.[1,69] It is important to appreciate that in such situations, where aphereses are carried-out daily, it is possible to lower this protein sufficiently to cause bleeding.[69]

The remaining clotting factors also drop between 25 and 50 percent of their physiological values and these low figures loosely correlate with large substitutions.[1,71,78,79] Data are available on II, VII, XI and XII with V and VII usually being fully corrected in 24 hours while the others typically remain reduced for twice this period.[79]

Conversely thrombosis may be predicated on the basis of significant decreases in antithrombin III[70] as well as in the protein C-S system although data for the latter is sparse. Less clear is evidence that qualitative haemostatic abnormalities play any clinically relevant role.

Immunoglobulins are frequently affected by plasma exchange[1,80] but this seldom translates into any problem with bacterial or other infections.

The complement system is made-up of a family of proteins which, once activated, convert C3 and C5 to the anaphylatoxins $C3_a$ $C5_a$.[76] The extent to which these changes occur is probably influenced by the nature of the artificial surface and the conditions under which the procedure was carried-out. Thus there is an important role for choice of anticoagulants, whether this be citrate or heparin, since the former, by chelating calcium, inhibits the conversion of inert to functional components.[76]

Because of these risks much research has been devoted to the fabrication of plasmapheresis membranes from synthetic polymers such as polypropylene and this technology reflects the world-wide importance attached to developing bio-compatible artificial surfaces in an attempt to reduce the generation of $C3_a$ and $C5_a$.[81]

In an early study with plasma separation achieved by centrifugation there was a decrease in total haemolytic complement or CH50 as well as the C3 and C4 sub-fractions. However all of these returned to normal within 24 hours but $C3_a$ and $C5_a$ were not directly measured.[1]

CONCLUSION

Long-term plasmapheresis is a well established and effective form of therapy in managing homozygous hypercholesterolaemia. The observation that anaemia occurs was interesting since it appears to have passed unnoticed other than for an oblique reference to the occasional individual who responded to iron. It is therefore particularly notable that the pathophysiologic mechanisms depleting body stores have attracted no attention. Since this is clearly of significance a prospective study was undertaken to define the incidence, characterise erythrocyte morphology and specifically explore aetiology. An additional objective was to establish whether any intervention was beneficial in preventing this complication and, if so, whether it had the potential to improve the quality of life.

CHAPTER IV

PATIENT PROFILES

TOTAL POPULATION

Heterozygotes

Seven representative individuals with familial hypercholesterolaemia were referred for plasma exchange between 1976 and 1993 (Table 3). This procedure did not consistently affect the lipid profile probably because they were treated erratically and only for short periods. Accordingly no further reference is made to data from these cases.

Homozygotes

Twelve patients were entered on protocol study during the same period. All had xanthomata of the Achilles tendon, interdigital cutaneous deposits, ejection systolic murmurs of supravalvular aortic stenosis whereas corneal arcus was present in all but three. An ileofemoral bruit was present in one. Three, presenting with angina pectoris, underwent coronary artery bypass grafting of whom one required an additional valve replacement. Family studies are available in six instances (Figure IIa, Figure IIb and Figure IIc).

Two of the 12 died from acute myocardial infarction and a third refused therapy (Table 4). Another three, who were eligible, formed a non-exchange control group because, one declined to participate, venous access was impossible in a second and severe ischaemia has required surgical intervention in the third (Table 4).

TABLE 3

HETEROZYGOTES

INITIALS	YOB	SEX	TC	HDL	LDL	TG	STATUS
D.E.	1948	F	15.13	1.0	13.46	1.45	LTF
A.J.	1937	F	9.14	.88	7.34	1.44	LTF
A.A	1944	M	11.01	1.0	9.21	1.75	DIED 10.3.88
A.Z.	1931	M	9.7	.9	6.75	.84	LTF
A.H.	1935	M	7.5	—	——	1.70	DIED 18-6-82
J.P.	1929	F	8.9	1.10	5.67	2.0	DIED 10-4-84
S.P.	1928	F	11.25	1.0	11.10	.90	LTF

Only the initial lipid data (mmol/L) is presented since these seven individuals were exchanged erratically and only for short periods. They are excluded from further consideration.

LTF = Lost to follow-up.

TC = Total cholesterol.

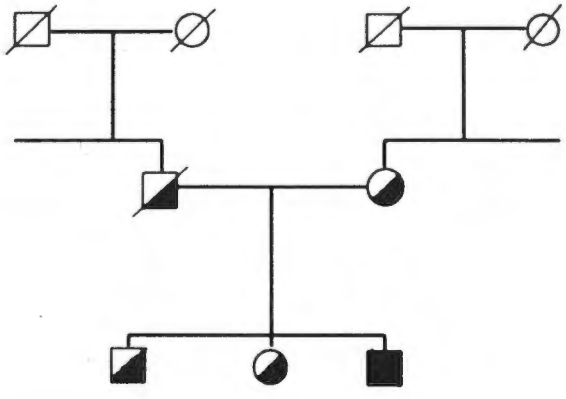
TG = Triglycerides.

YOB = Year of birth.

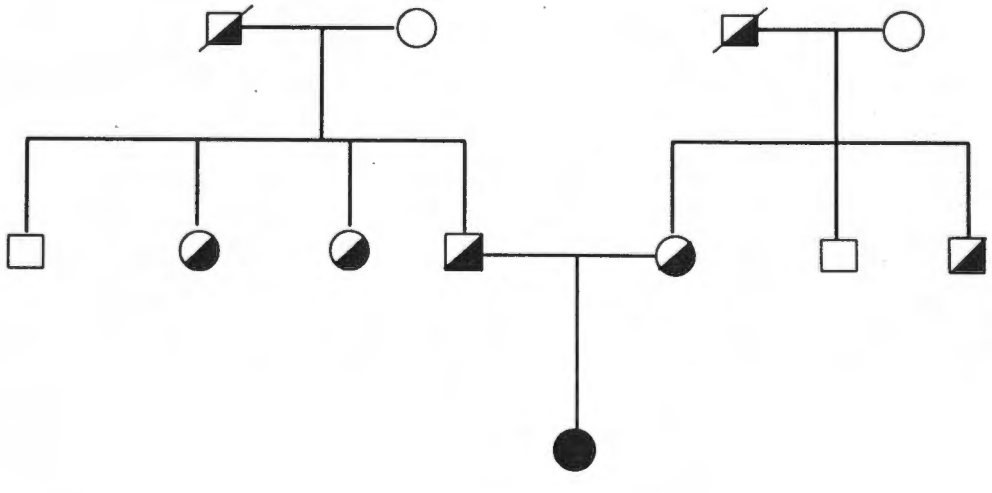
FIGURE IIa

FAMILY TREE

G.v.d.M



A.C.

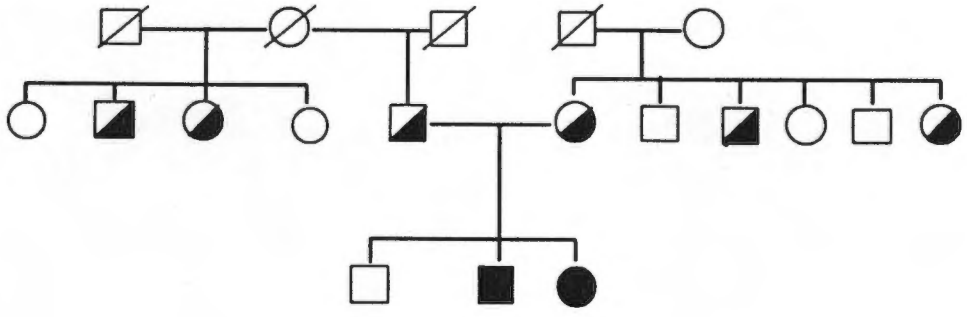


In these three consecutive illustrations lines extending beyond the symbol signify death of that person. Solid shading denotes homozygosity and half-filled squares or circles are the heterozygotes. Open symbols reflect uninvolved individuals. Bisected symbols mean that no data is available.

FIGURE IIb

FAMILY TREE

Y.A./S.A.



C.F.

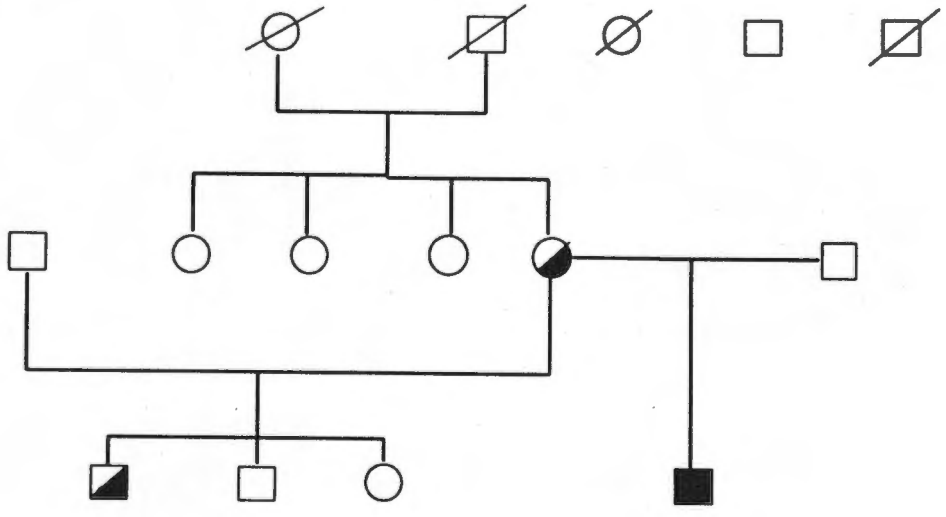
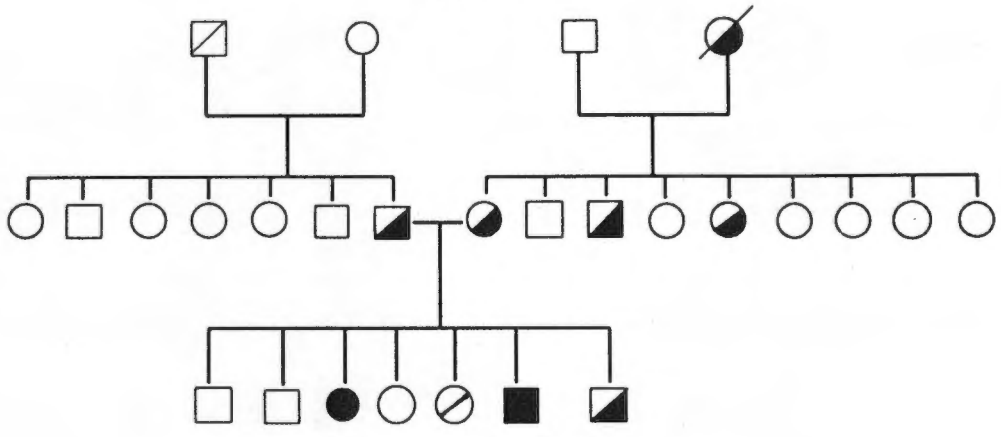


FIGURE IIc

FAMILY TREE

CI/RI



P v.D

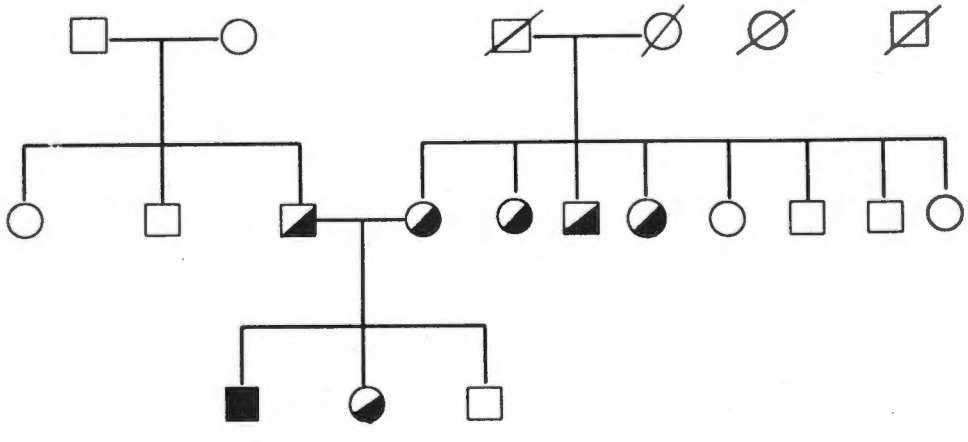


TABLE 4

HOMOZYGOTES - REFERENCE GROUP

INITIALS	DOB	SEX	TC	HDL	LDL	TG	STATUS
N. v.d. M	15-1-74	F	21.92	0.68	20.57	1.5	DIED 2-4-86
P. v.d. D	9-11-70	M	18.20	0.73	16.68	1.72	REFUSED 19-4-93
S.A.	31-3-73	M	22.82	0.71	13.57	0.61	DIED 15-2-90
E.S.	26-5-70	F	9.9	1.2	8.4	.6	Alive
T.D.	25-12-81	F	24.2	.3	23.4	1.0	Alive
R.I.	9-11-69	F	17.4	.9	16.2	.6	Alive

Initial lipid data (mmol/L) are available in these six patients. The first three participated only briefly whilst the remainder were excluded for the reasons given.

STUDY GROUP

The demographic data on the remaining six is given in Table 5. Prior to January 1991, (Phase 1) all had failed protracted periods of lipid-lowering agents that routinely included questran, clofibrate, probucol and bezalip often in addition to maximally tolerated doses of nicotinic acid. During that year (Phase II) changes in the lipogram were defined in response to plasma exchange alone as a control period for the simvastatin trial (Phase III) completed at the end of 1992: these results have been reported.[10,11] The current study (Phase IV) undertaken to systematically investigate the pathogenesis of anaemia has occupied 1993 and 1994.

TABLE 5

HOMOZYGOTES - STUDY GROUP

INITIALS, DOB AND GENDER	LDL RECEPTOR AND MUTATION	TC, LDL, HDL, TG	Fe, TIBC, % SAT., FERRITIN	ANGIOGRAPHS	FISTULA INSERTION AND PE COMMENCED
G v d M 7-2-62 M	LOW AfrFH1/AfrFH2	18.98 NA NA NA	NA NA NA NA	1. Trivial coronary artery disease with single area of 40% narrowing in mid-LAD artery. 2. Trivial aortic stenosis (PSG = 8mmHg) 1988: coronary artery bypass	1. 12-2-90 2. 17-2-90 3. 07-3-91 4. 13-7-92 5. 19-9-94 20-04-76
A C 2-8-70 F	HIGH AfrFH1/AfrFH1	17.26 NA NA NA	NA NA NA NA	Critical narrowing of (L) coronary artery. 1979: Saphenous vein bypass graft from aorta to LCA.	8-1-79 05-12-78
Y A 15-3-76 F	HIGH AfrFH3 + OTHER	19.7 17.22 0.81 0.90	11.1 66.1 20 38	Normal: no cardiac lesion	6-9-86 23-10-86
C F 21-9-75 M	LOW AfrFH1/OTHER	18.12 16.59 0.62 1.99	11.7 65.2 18 21	No significant coronary artery disease	10-2-86 05-03-86
C I 12-1-61 M	NEGATIVE Cape Town2/ Cape Town2	15.05 13.89 0.59 1.25	9.9 67.0 18 40	Normal	2-3-89 27-04-89
J G 16-6-71 F	HIGH AfrFH1/AfrFH1	18.20 17.40 0.80 0.60	11.0 61.0 18 37	Mild supravalvular aortic stenosis. Insignificant single vessel coronary artery disease. 15-3-74: Porto-caval shunt.	8-2-91 05-03-91

These six cases were serially studied over the two year period of this investigation. The initial lipid data (mmol/L) is given in this table whereas the serial values, in response to plasma exchanges, have been previously reported.[10,11]

The designation of defective receptor status as negative, low or high is based on skin fibroblast LDL binding studies in comparison with controls and refers respectively to less than 4%, 5-20% or 20-30%.[11]

CHAPTER V

PRINCIPAL METHODS

APHERESIS PROCEDURES

Introduction

The treatment protocol was approved by the Ethics and Research Committee of the University of Cape Town and Groote Schuur Hospital with participation requiring informed consent and, in the case of minors, this was obtained from their legal guardians. These cholesterol-depleting exchanges were scheduled every alternate week, using a vein-to-vein circuit, and replaced one and a half times the calculated plasma volume on each occasion. Non-invasive cardiovascular monitoring (Sirecust System, model 404-1A, Siemens Medical Electronics Incorporated, Danvers, Maryland, USA) and maintenance of a written and detailed work record was routine. At completion any cellular components trapped within the disposable lines were flushed back into the patient using small quantities of physiological saline. After withdrawal of the needles pressure was applied to the venipuncture sites to prevent bleeding.

Centrifugal Separation

The Cobe, model 2997 (Cobe Laboratory International, Lakewood, Colorado, USA) continuous-flow instrument, has an extracorporeal capacity of approximately 280 mL and incorporates safety features in the form of access and return monitors as well as air detectors.55,82

Air was displaced from the tubing by priming, over a 30 to 40 minute period, with 0.9 percent sodium chloride (Adcock Ingram, Johannesburg, South Africa) to which was added acid-citrate dextrose solution USP formula A (ACD-A): (Baxter

Health Care Corporation, Fenwal Division, Deerfield, Illinois, USA) in a ratio of 10 to 1.

Venous blood, at a flow rate between 40 - 60 mL per minute, is pumped into the single-stage band (Figure III) rotating at a speed of 1400 revolutions per minute with cell separation being achieved in approximately 2 minutes. At this point plasma is diverted to a discard bag and isovolaemic replacement commenced with a 5 percent albumin solution (20 grams percent salt-free human albumin: Western Province Blood Transfusion Service, Cape Town, South Africa) in plasmalyte B (0.9 percent saline containing potassium, magnesium and bicarbonate: Baxter-Travenol Laboratories, Deerfield, Illinois, USA). To avoid changes in body temperature an in-line warmer (Model 4R 4306, Fenwal, Baxter-Travenol Laboratories, Deerfield, Illinois, USA) was employed.

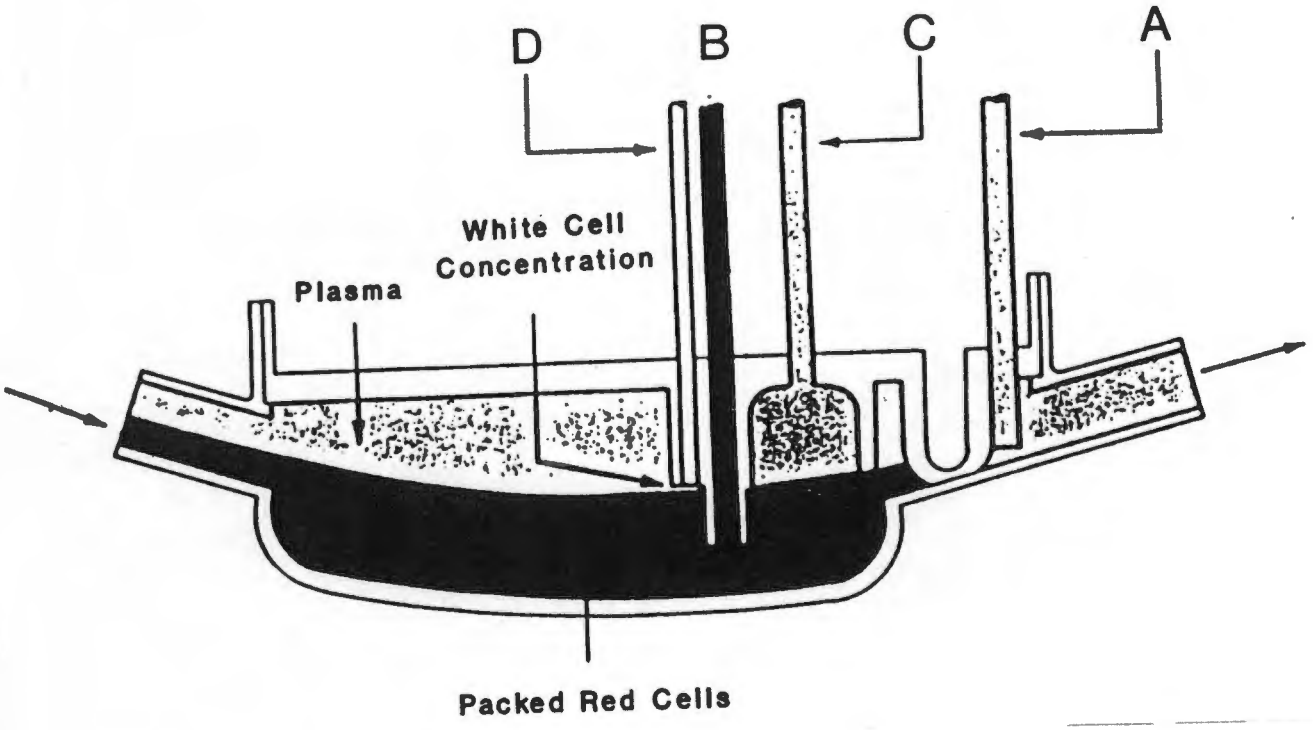
Anticoagulation was maintained by a monitored addition of ACD-A at a ratio of 1:11 at the site of the collection needle and this step was checked by a flow indicator. A small citrate effect occurred in the patients as reflected in an asymptomatic drop in ionisable calcium.[3] Significant hypocalcaemia was prevented by prophylactic infusion of 10 mL of 10 percent calcium gluconate (Labethica, Cape Town, South Africa) for each litre exchanged and this was given at a rate of approximately 1 mL for every five minutes of the procedure and corresponds to 3.75 mEq.

Filtration Technique

The Cobe Centry TPE (Cobe Laboratory International, Lakewood, Colorado, USA) operates on the principle that formed

FIGURE III

SINGLE STAGE BAND



Anticoagulated whole blood enters the band (A). Once separation has occurred packed red cells are returned to the patient (B) and plasma is diverted (C) with isovolaemic addition of exchange fluid added to the return line. The white cell collection port (D) is inactive during plasmapheresis.
In this instrument the cells are separated from plasma in a centrifugal field.

elements are retained by a microporous membrane (Figure IV). An extracorporeal space of only 150mL is well suited to management in small individuals.[82] Safety features include air detectors and a device to monitor transmembrane pressures. A useful indicator is that for displaying the ratio between volumes removed and replaced thereby ensuring that they accurately match. With such flexibility it is simple to achieve controlled degrees of under or over-hydration. Priming takes about 10 minutes using saline and ACD-A at a ratio of 25 to 1.

The procedure is initiated at a flow rate of 20 mL per minute and is gradually increased two or three fold with plasma separation determined by this variable and the packed cell volume.

Anticoagulation is started with 50 international units per kilogram of heparin (Intramed (Pty) Limited, Port Elizabeth, South Africa) and, depending on the duration of the run, may be repeated although not in the last 60 minutes. Additionally ACD-A is continuously infused at a ratio of 1:25 to whole blood: any untoward citrate effect is counteracted by incorporating 6 mL of 10 percent calcium gluconate in each litre of replacement fluid corresponding to 2.25 mEq of calcium.

LOW DENSITY LIPOPROTEIN RECEPTOR STATUS

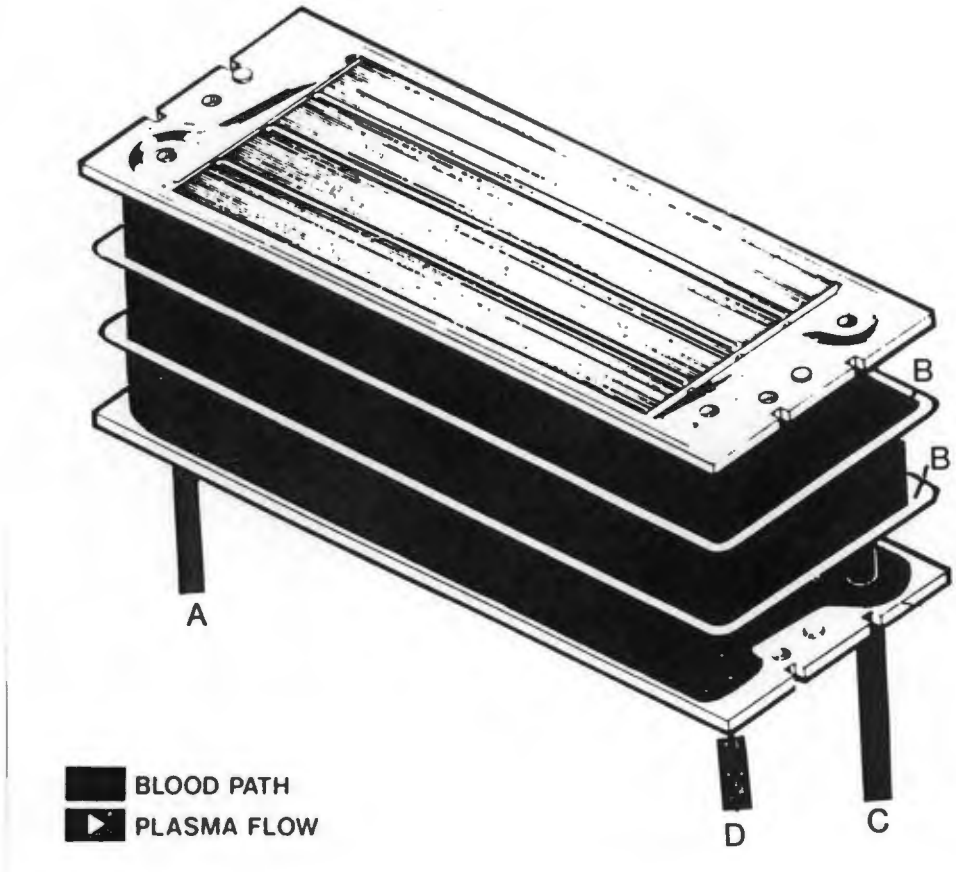
Homozygosity had previously been established in all cases on standard clinical criteria, binding studies[83] and with the mutations in the coding sequence defined.[9]

LIPOGRAMS

These were serially determined before and after each

FIGURE IV

THE MICROPOROUS MEMBRANE FILTER



Venous blood is pumped into one side of the system (A), circulates between parallel membranes (B). Packed red cells are returned to the patient (C) and plasma discarded (D).

apheresis and also on the discard bag. Cholesterol was enzymatically measured using an esterase to hydrolyse the esters and to assay hydrogen peroxide produced by oxidation of the free lipid (Cholesterol Liquicolor Kit, Human GmbH:W6204-Taunusstein 4, Germany); high density lipoprotein cholesterol using the same method after precipitation of other proteins by polyethylene glycol (Trace HDL-cholesterol Kit, Trace Scientific [Pty] Limited, Melbourne, Australia); triglycerides by enzymatic hydrolysis of lipase followed by assay of glycerol (Triglyceride GPO-PAP Kit, Boehringer Mannheim and Company, GmbH:Mannheim, Germany). LDL cholesterol was then calculated by subtracting HDL and triglyceride values from total cholesterol.[84]

The kinetic pattern at which lipids return to their pre-exchange levels was determined by repeating these measurements on four consecutive days in the week of apheresis and three of the following seven days.

THE BLOOD COUNT

This was carried-out using an automated particle counter (Technicon H1, Tarrytown, New York, USA) and comprised haemoglobin, total erythrocytes, leucocytes and platelets together with differential spread. In the context of this study particular attention was paid to the red cell indices in the form of mean corpuscular volume (MCV) and haemoglobin (MCH) as well as concentration (MCHC).[85]

ANALYSIS OF IRON INTAKE

Dietary composition of each meal was calculated using portion size, food composition tables,[86] a standard quantities manual[87] and an appropriate computer programme with software

(Food Finder, version 1:10, Medical Technologies [Pty] Limited, Kuils River, Cape South Africa). This was extrapolated to the daily intake and in turn, to ingestion averaged over the 28 day study period. Consideration was concurrently given to the presence of promoters such as ascorbate or inhibitors including tannate, phytates or phosphate that might qualitatively enhance or inhibit availability of this trace metal.[88,89]

EXCLUSION OF BLOOD LOSS

In females it was established, by carefully taking menstrual history that, from menarche to the current time, there had been no significant changes. In none of the participants had there been abdominal symptoms, alteration in bowel habit or colour of stools other than when these became dark during periods on oral iron replacement. Iatrogenic blood loss was calculated from sample collections.

NUTRITIONAL STATUS

Iron

Serum levels and binding capacity were based upon colorimetric techniques as recommended by the International Committee for Standardisation in Haematology ICSH[90,91] and percentage saturation then calculated. Serum and red cell ferritin, reflecting stores were assayed immunoradiometrically by reaction with radio-labelled antibody and removal of excess by immunoadsorption using a commercial kit (Becton Dickinson and Company, Orangeburg, New York, USA).[92,93] The same methods were applicable to discard fluid where controls were aliquots collected prior to the exchange.

Vitamin B_{12} and Folate

These were simultaneously quantitated in plasma (SimulTRAC-SNB Radioassay Kit; Becton Dickinson and Company, Orangeburg, New York, USA).

SCREENING TESTS FOR HAEMOLYSIS

Serum lactic dehydrogenase and isoforms were measured by agarose electrophoresis (Paragon LD- Electrophoresis Kit; Beckman Instruments Incorporated, Fullerton, California, USA) and plasma haptoglobin by nephelometry (Behring werke, Marburg, Germany).

RADIONUCLIDE INVESTIGATIONS

Red Cell Mass and Survival

These were determined on day zero and 14: in each case immediately prior to consecutive plasmapheresis; only minor modifications were made to standard methods.

Day 0

Red cell mass was measured as the patient came off the machine by injecting an aliquot of autologous venous blood that had previously been collected into ACD-A and labelled with 1.11 MBq (30μCi) of ^{51}- radiochromium.[94,95]

Survival was carried-out over four weeks following the course of the same radiochromated erythroid population.[96] In practical terms accurately pipetted 2 ml samples, collected daily for the first and third week and alternate days for the second and fourth were lysed with saponin and the ^{51}Cr detected with appropriate window settings on a auto-gamma counter. Using these points, correction was made for elution with a computer programme[97] and a variety of curve-fitting statistics.[98]

Day 14

Red cell mass was remeasured using 74 MBq (2mCi) of ^{99m}Tc-label (Ultra Tag RBC Kit: Mallinckrodt Medical, Code DRN 4350) added to 3 ml venous blood in 1 ml ACD-A. Prior to administration the cells were washed twice in saline according to ICSH recommendations,[95] 2 ml aliquots were obtained at 10 and 20 minutes after infusion and analysed with automatic correction for ^{99m}Tc-decay and zero-time activity derived by linear extrapolation on a semi-log plot.

Erythrocyte Survival by Urinary Radioisotope Excretion

Red cell life-span was independently estimated by determining loss of radiochromium in the urine[99,100] with 24 hour collections made on seven consecutive days of week one and similarly week three. Duplicate samples were counted against a standard derived from a known dilution of the orginally chromated erythrocytes with appropriate computer fitting of exponential values without taking elution into consideration.

Plasma Volume

This was determined immediately before and after plasmapheresis using 0.185 MBq (5μCi) of iodinated (^{125}I) human albumin on each occasion according to established procedures.[95] Correction was made for background radioactivity by counting a sample collected before each injection. Ten and 20 minute points were extrapolated back to zero-time on a semi-log plot.

Total Blood Volume

This was calculated prior to day 14 apheresis as the sum of red cell mass and plasma volume using ^{125}I-human albumin and ^{99m}Tc-red cells.

Blood Loss During Plasmapheresis

The 2 to 4 litre of discard plasma was, in each instance, well mixed and two 10 ml aliquots pipetted into screw-cap plastic vials: samples of fluid collected from the extracorporeal circuit was treated similarly. These specimens were then counted against an appropriate ^{51}Cr-RBC reference as described above. All disposable plastic lines and residual fluid recovered at the completion of the run were counted in a suitable instrument with appropriate settings and controls.

From these three sets of data the percentage radiochromated erythrocytes injected on day 0 and lost during the day 14 procedure could be quantitated.

Red Cell Retention

Following plasma exchange apparent retention of ^{99m}Tc-red cells injected on day 14 was determined by counting 10 and 20 minute aliquots against appropriate standards.[94]

Modifications for Non-exchanged Patients

Day 0

Red cell mass and four week radiochromium survival, as well as urine-based determination, were performed as above except that no delay in re-injection was necessary to accommodate the exchange.

Day 14

Red cell mass was as outlined but repeat plasma volume not carried-out. The second urine-based radiochromium measurement was performed as previously described.

ERYTHROPOIETIC RESPONSE

The functional integrity of the erythron was evaluated by reticulocyte counting and determination of the maturation index by flow cytometry,[101] glucose-6-phosphate dehydrogenase (Sigma, procedure No. 345-UV: Sigma Chemical Company Limited, Poole, Dorset, England),[102] and pyruvate kinase (MPR 1 Pyruvate Kinase Kit; Boehringer Mannheim, France),[103] with erythropoietin directly measured by radioimmunoassay.[104]

EKTACYTOMETRY

Red cell deformability, in response to known rheological stress, was determined under a variety of conditions using a concentric cylinder viscometer of the couvette type (Technicon Ektacytometer, Saint-Dennis, France). Based on the method originally described by Bessis and Mohandas[105] where particles were suspended in a viscous medium and where revolution of the inner cylinder creates a shear field there is alignment and elongation which is quantitated by a laser beam that produces a coherent light-scattering pattern or defraction image.[106] The end points are seen in the osmoscan which defines the behaviour of intact erythrocytes to a solution of gradually increasing osmolality within the viscometer as an index of deformability of the entire cell including membrane and contents.[107,108] Secondly, deformability, as well as fragility, is recorded by scanning ghosts or free membranes suspended in dextran: curves are generated as forces increase

with revolutions going from zero to 150.[109,110] In recognition of the fact that iron deficiency may influence these same measurements each of the studies was repeated after stores had been returned to normal by therapeutic courses of ferrous gluconate.[111]

IRON EXCRETION

This trace metal was quantified in the discard fluid, together with the range of other elements, using standard inductively coupled plasma atomic emission spectroscopy (ICP-AES).[112] Total volumes were noted and aliquots then digested after which the sample was atomised into an argon plasma and quantitated using high-quality grating spectrophotometer for ultraviolet and visible regions with a photomultiplier detector (The Jobin-Yubon ICP Machine, model JY 70 C-plus Jobin, Paris, France). The advantage of the system is that no elaborate preparation is required while simultaneous measurement of a number of substances can be achieved with high degrees of sensitivity and accuracy. Importantly the technique is relatively insensitive to matrix composition and the spectrometer can readily be coupled to a high performance liquid chromatograph, an FIA device or electrothermal atomiser.[113] Haemosiderin was determined in the urine using standard methods.[114]

CHAPTER VI

ADDITIONAL METHODS

VISCOSITY

In the plasma viscosity was measured by timed passage through a narrow capillary tube (Viscometer 2, Coulter Electronics Limited, Northwell Drive, Luton, Beds, England).[115] The whole blood measurements employed a cone-plate viscometer (Wells-Brookfield Engineering Laboratories Incorporated, Stroughton, Massachusetts, USA).[116]

GRANULOCYTOPOIESIS AND NEUTROPHIL FUNCTION

Total white count and differential were carried-out before and after apheresis using the Technicon H1 Automated Particle Counter.

Superoxide anion was measured after stimulation with phorbol-myristate-acetate by reduction of ferricytochrome c to ferrocytochrome c and the colour change determined spectrophotometrically.[117] Nitroblue tetrazolium was quantitated in neutrophils after exposure to endotoxin with conversion of the colourless dye to a detectable formazan.[118]

The phagocytic index was defined by ingestion of heat-killed candida albicans spores.[119] Bactericidal capability was evaluated by the ability of neutrophils to ingest and kill staphylococcus aureus[120] with a modified end point being thymidine incorporation. Chemotaxis and random migration was determined using a standard agarose method.[121]

LYMPHOCYTE IMMUNOPHENOTYPING

At six monthly intervals pre-exchange pattern was retested using a standard panel of monoclonal antibodies comprising CD2, 3, 4, 8, and 19[122] by means of indirect

immunoflourescent microscopy (Nikon Optiphot Labophot Episcopic-Fluorescence Attachment "E,F"/"EPA" Nippon Kogaku K.K., Tokyo, Japan).[123]

THROMBOCYTOPOIESIS AND PLATELET FUNCTION

Bleeding times could not routinely be included because of patient non-compliance. Aggregometry was carried-out before and after plasma exchange under standardised conditions in a twin-channel instrument (Cronolog Corporation, Wholeblood Aggregometer Model 560-VHS Linked to a Strip-chart Recorder, Model 707, Haverton, Pennsylvania, USA). Patient response was compared to normals using adenosine diphosphate in concentrations of 0.1, 0.01 and 0.005 molar (Sigma Chemical Company, St Louis, Missouri, USA), collagen 15 mg/ml (Sigma Chemical Company), epinephrine or adrenaline 0.01 molar (Maybaker [SA] [Pty] Limited, Holland Park, Port Elizabeth, South Africa), ristocetin 1.5 mg/ml (Sigma Chemical Company).[124,125]

COAGULATION PROTEINS AND COMPLEMENT

Methods were those that have previously been calibrated and described.[1] These included prothrombin and partial thromboplastin time according to standard techniques,[126] fibrinogen,[127] immunoglobulin,[128] complement and immune complexes,[129] antithrombin III[130] and factor eight.[131]

BIOCHEMISTRY

All parameters were measured on an automated system (Hitachi 747 Automated Discreet Analyser, Boehringer and Mannheim Limited, Tokyo, Japan) with sodium, potassium and chloride based upon ion-specific electrodes. The remaining chemistries were determined by either calibrated enzymatic or

calorimetric methods using commercial kits (Boehringer and Mannheim Limited, Tokyo, Japan). Ionizable calcium were carried-out as reported (A.V.L. 9875, Beckman, South Africa).[3]

CHAPTER VII

MAJOR RESULTS

APHERESIS PROCEDURES

Further experience accumulated during this period underlines the safety of both centrifugal and filtration techniques.[10,11] Throughout these investigations changes in plasma components, such as the lipids, directly related to any procedure were documented and they show no deviation from those reported previously.[11] Of note is the ability to achieve the desired effect on LDL cholesterol efficiently and without loss of any of the surgically created arterio-venous fistulae. Of passing interest is the fact that this generation of separators is now obsolete. Those with a reusable polycarbonate bowl have been replaced by the Cobe Spectra. However the therapeutic plasma filtration unit has been retained for managing small individuals with their limited tolerance for extra-corporeal sequestration of effective plasma or whole blood volume.

LOW DENSITY LIPOPROTEIN RECEPTOR STATUS

Although not central to this long-term apheresis aspect of managing these patients it is instructive to note that, of the heterozygotes (Table 3) who are slightly older although with less severe cholesterol disturbances, half have died at relatively early age.

The six non-study homozygotes (Table 4) have essentially the same biochemical defect as the plasma exchanged group and the lethality of the vascular disease is reflected in their early death rates.

In considering the study cohort (Table 5) all but one are seen to be Friedrickson type IIA. Half these cases exhibit homozygosity for the Afrikaner mutation where fibroblast cultures showed impaired binding of low density lipoprotein to the cognate receptor that, in turn, resulted in the characteristic hyperlipidaemia. A point of some interest, presently unexplained, is that the molecular defect does not universally correlate with the phenotype in which there are high levels of total as well as low density lipoprotein cholesterol.[33a] The degree of ligand attachment, as this might influence availability for clearance during the exchange procedure, was not specifically investigated.

LIPOGRAMS

Effect of Creating Arterio-venous Fistulae

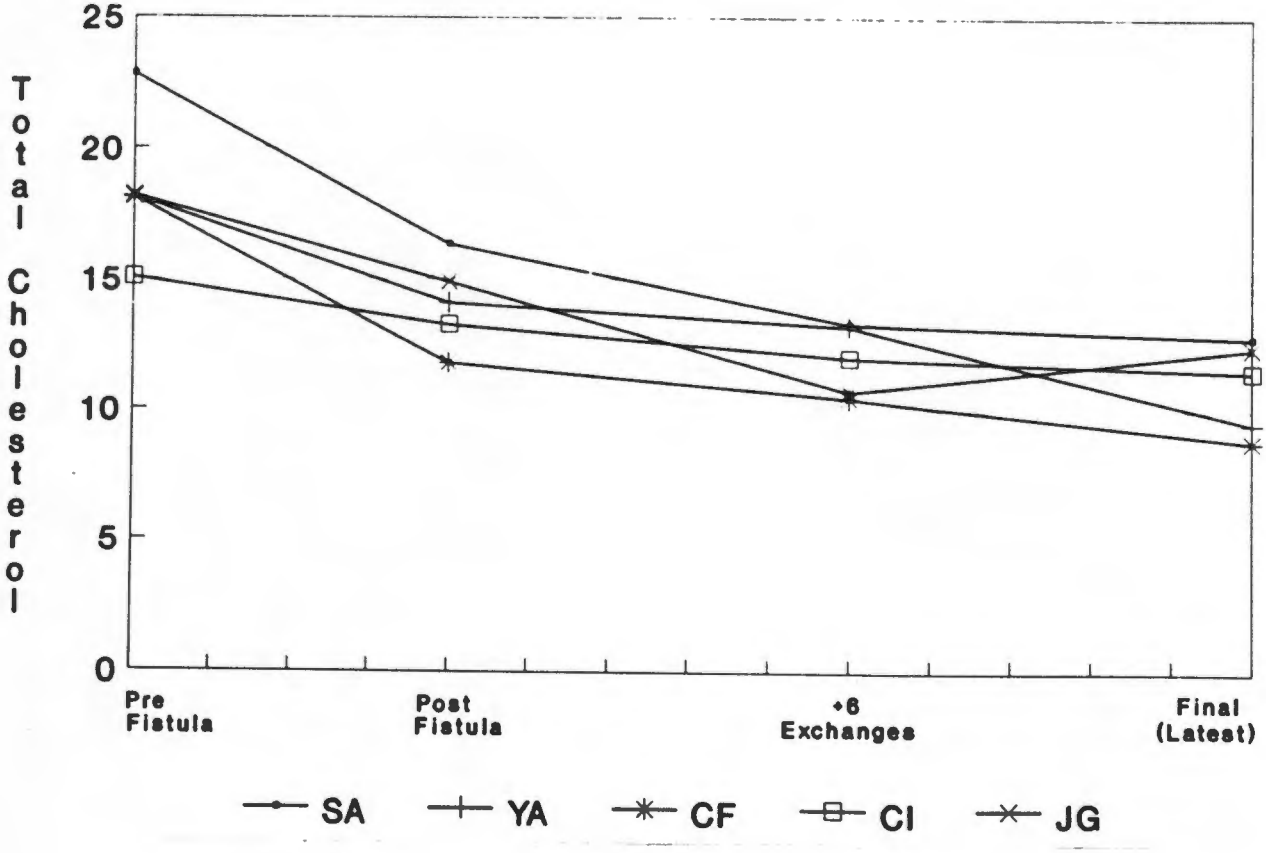
Inexplicably total and LDL cholesterol marginally, although significantly, dropped prior to commencing plasmapheresis: this was not noted with triglycerides or the HDL fraction (Figure V).

Impact of the Apheresis Procedure

Mean and standard deviation for total cholesterol fell from 12.1 (0.9) to 3.9 (0.6) with the corresponding values for low and high density lipoprotein fractions being, respectively, 10.8 (0.9) to 3.4 (0.5) and 0.7 (0.1) to 0.3 (0.1): the triglycerides decreased from 1.0 (0.4) to 0.5 (0.1) (Figure VI). This is a consistent variation being most striking for total and LDLC. Unfortunately there was also a reduction in HDL which was better preserved using affinity column apheresis and therefore the latter has an advantage in terms of a greater protective effect.[49]

FIGURE V

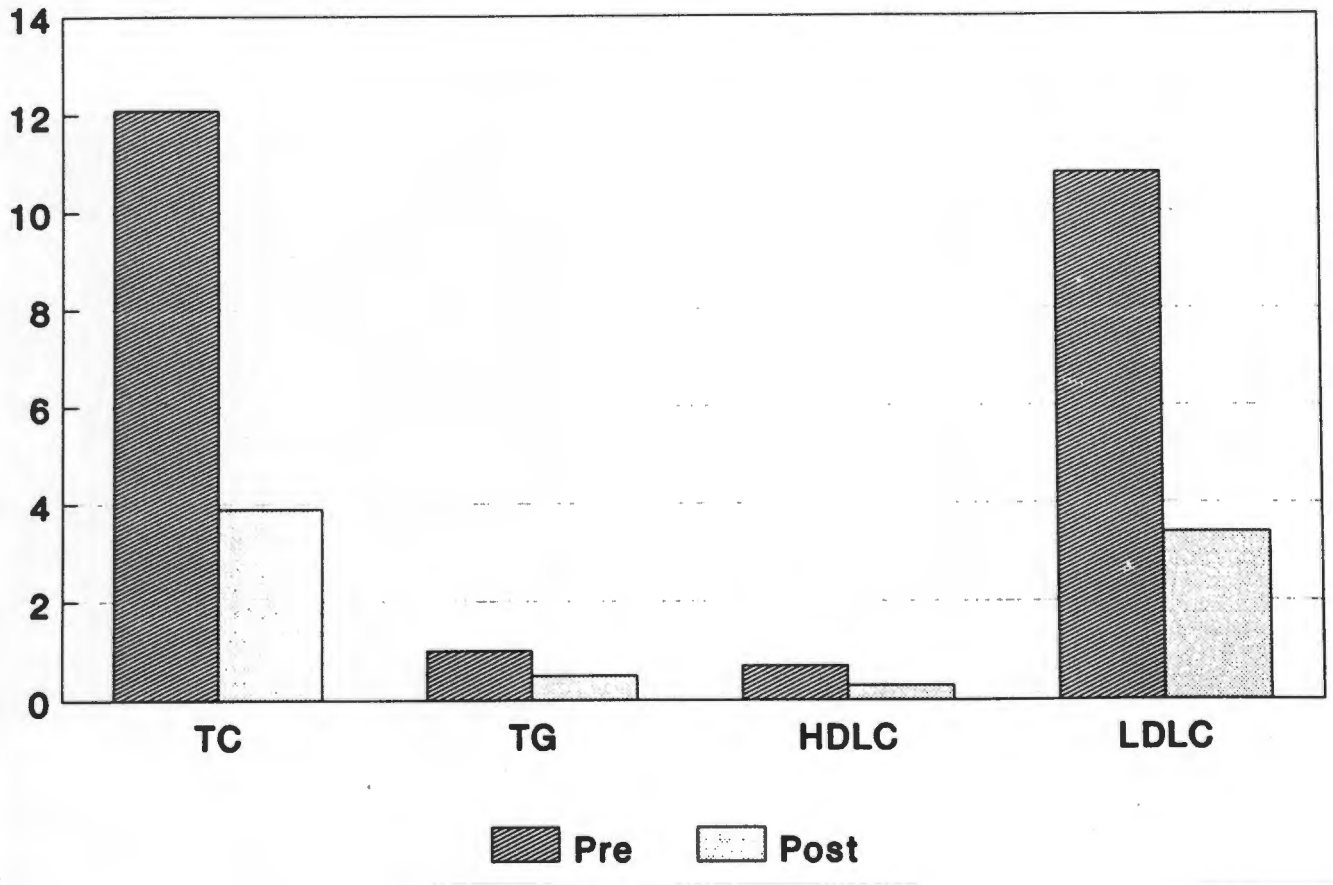
THE EFFECT OF CREATING AN ARTERIO-VENOUS FISTULA



Inexplicably total cholesterol dropped after the procedure in all five patients as illustrated: a similar change was found with the LDL fraction but this is not shown. Of note is the observation that neither HDL nor triglycerides fell and, accordingly, these changes in levels are not shown. The vertical axis is total cholesterol in mmol/L: the horizontal axis is an arbitrary scale and not in months.

FIGURE VI

IMPACT OF THE APHERESIS PROCEDURE



Mean values show a striking drop with each procedure for all fractions. However, the fact that this extends to HDL reduces the benefit of lowering total and LDL cholesterol.[11]

Post-exchange Lipid Kinetics

In the short term lipid concentrations are seen to rise and, whilst there may be some contribution from extravascular compartments, the preferred explanation is that this reflects secretion of newly made lipoprotein from the liver. Examined regularly these have returned to the pre-apheresis levels by day 14 (Figure VII). It follows that, to sustain maximally effective reductions, plasmaphereses should be carried-out at one rather than two week intervals. This recommendation assumes that more efficient clearance achieved with the intensive schedule will impact positively on cardiovascular disease but this remains an unproven contention.

Nevertheless, even once every 14 days was sufficient to improve plasma levels over the commencing value but, after a three month period a stable plateau developed - as previously reported.[11] It is appreciated that this schedule reflects a pseudo-equilibrium and may not reach the concentration that is genetically governed.

THE BLOOD COUNT

Haemoglobin and Packed-cell Volume

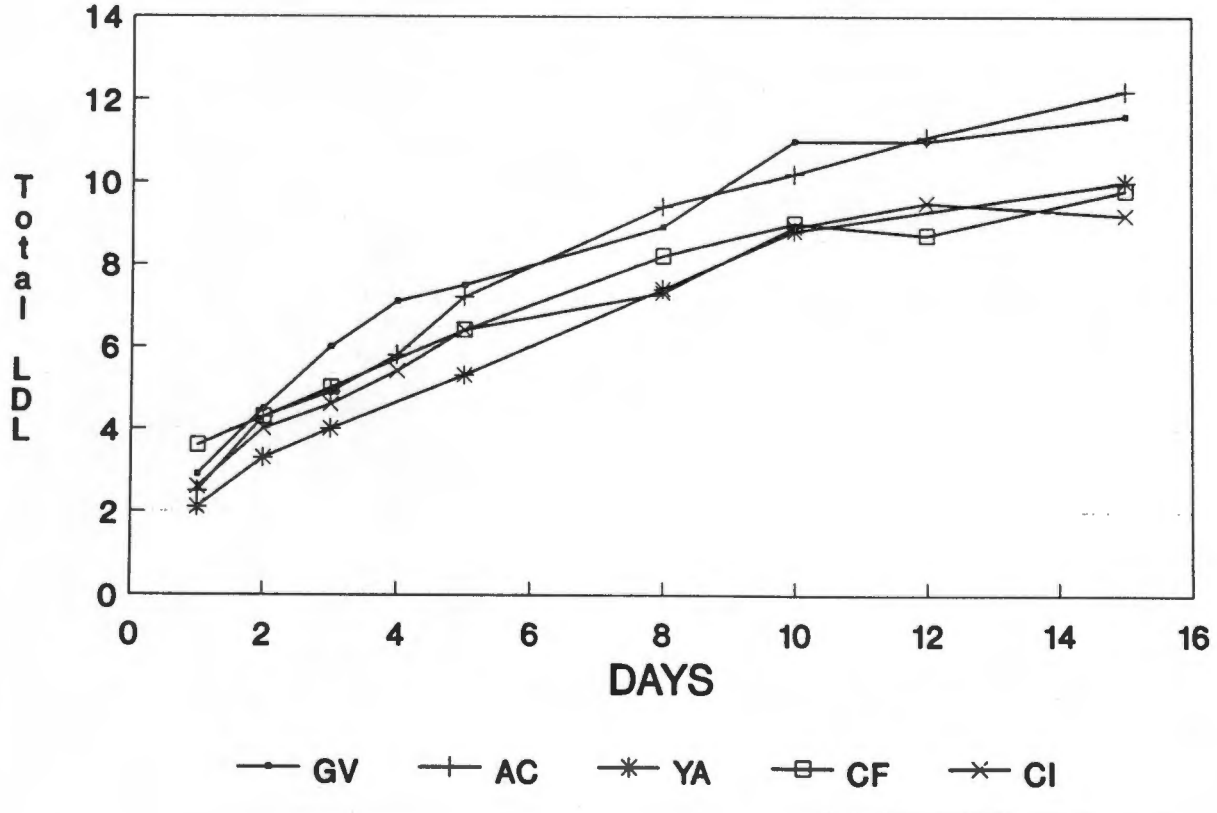
Both showed a statistically significant downward trend when plotted against time or number of exchanges. This was sharply reversed either by infusion of intravenous iron dextran or oral administration of 110 mg of ferrous gluconate three times a day. Conversely where neither intervention took place the steady deterioration continued (Figure VIII).

Red Cell Indices

A comparable response occurred in both mean cell volume and haemoglobin as well as mean cell haemoglobin

FIGURE VII

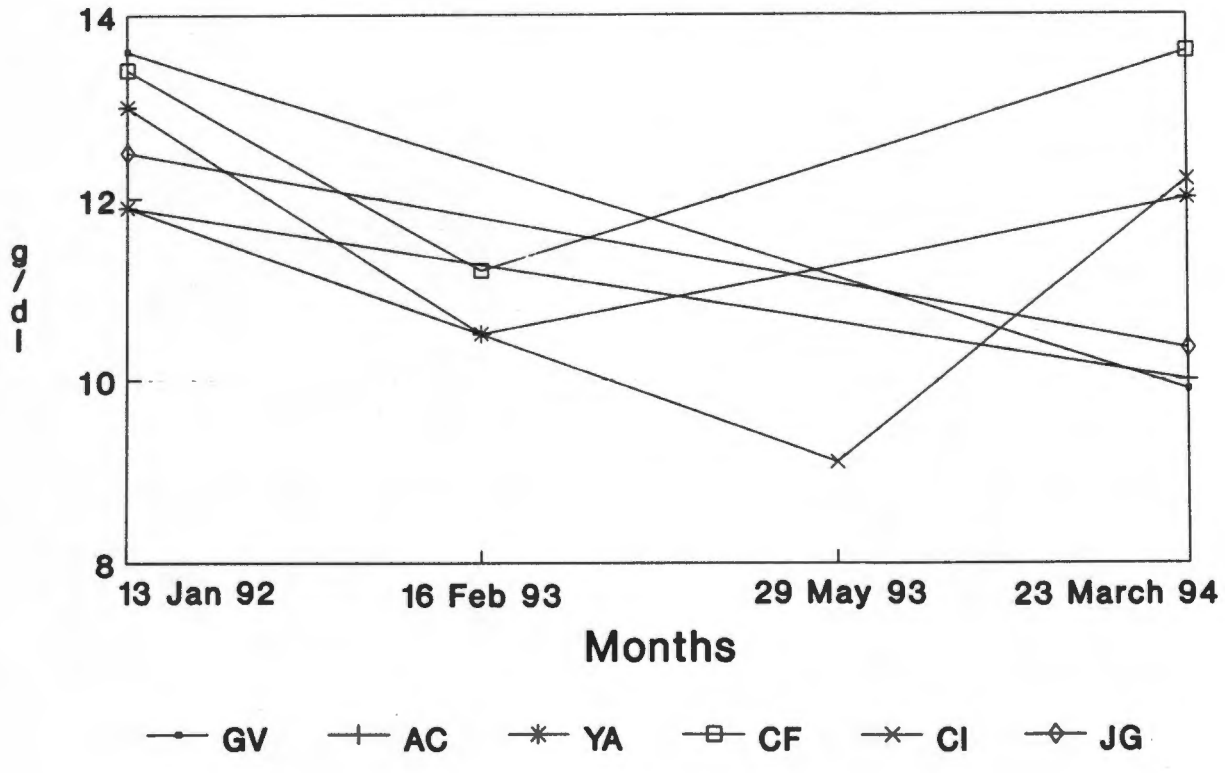
POST-EXCHANGE LIPID KINETICS



Low density lipoprotein rose gradually and had returned to pre-treatment levels over a two week period. Similar patterns occurred with total cholesterol HDLC and triglycerides (data not shown). On the vertical axis LDL cholesterol is expressed in mmol/L.

FIGURE VIII

HAEMOGLOBIN AND PACKED-CELL VOLUME



The vertical axis shows haemoglobin in grams per decilitre: parallel data from PCV are not shown. Of the six patients those who received no iron had a continued drop in haemoglobin level. In contrast oral or parenteral replacement leads to prompt return to normal.

concentration (data not shown).

ANALYSIS OF IRON INTAKE

Hypochromic and microcytic erythropoiesis acquired during the observation period focused attention on the need to document adequacy of iron intake. It was demonstrated, probably as a result of the low-fat diet being ingested, that absolute quantities of iron were below the recommended daily allowance except in one patient (Figure IX). Since the latter measurements are generated for a normal population it follows that they are not strictly applicable to anaemic patients.

Apart from the patients taking in less red meat than the remainder of the family the composition was qualitatively normal with neither increase or decrease in currently recognised promoters or inhibitors such as tannates, phytates or phosphates being present.

EXCLUSION OF BLOOD LOSS

Conversely collection of routine blood samples, even when corrected to the average anaemic haemoglobin level of these patients, accounted for a minimum loss of 17 mg per month. Over long periods of time no gastrointestinal or genitourinary tract symptoms or signs occurred. Patients young age and natural reticence precluded direct measurements on the stools.

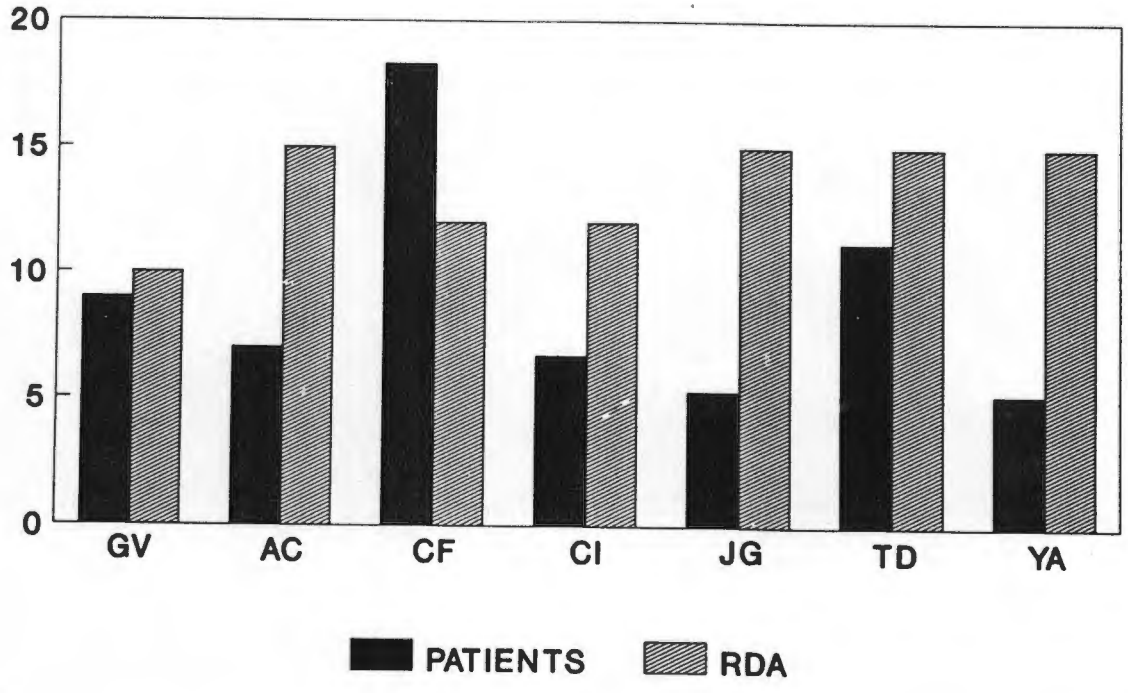
NUTRITIONAL STATUS

Iron

Gradual depletion of body iron stores was reflected in falling serum levels of this trace metal accompanied by rising total transferrin so that percentage saturation (data not shown) of the latter iron-transporting protein dropped statistically with time. Essentially a similar pattern was

FIGURE IX

ANALYSIS OF DIETARY INTAKE



These intakes, shown in mg/24 hours on the vertical axis, are without iron supplementation and, with a single exception, all study patients are seen to take in less iron than their recommended daily allowance: this reflects the low-fat diet ingested by these individuals. It is acknowledged that RDA is generated for a normal population and therefore not directly applicable to patients with iron deficiency anaemia.

found with mean serum ferritin dropping from 28 to 10 ng/mL. Theoretically these iron-binding proteins might be spuriously depressed from losses in the exchange procedure but this possibility was not specifically documented. In addition, depletion of transferrin could not be predicted from determining the albumin level, since the latter was supplied in the exchange fluid. Nevertheless the changes in these iron containing proteins correlated with the appearance of hypochromasia and microcytosis and appeared to be related to the development of iron deficiency. These abnormalities reversed promptly with replacement therapy.

Vitamin B_{12} and Folate

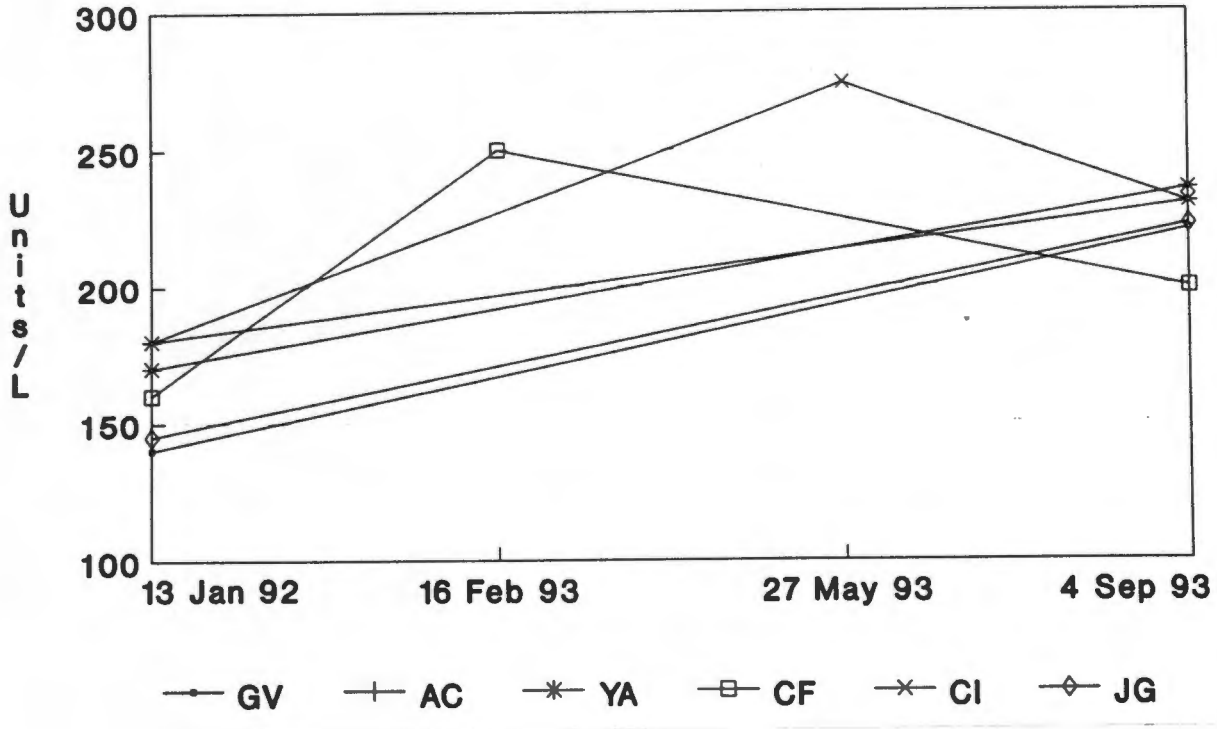
Neither of these essential haematinics varied at any stage during the study.

SCREENING TESTS FOR HAEMOLYSIS

Recognising that major nutrients for blood formation were approximately balanced, albeit iron only marginally in terms of sub-optimal quantitative intake, its additional loss possibly due to intravascular haemolysis, was examined. Here a significant trend for increase in lactic dehydrogenase was noted although red cell isoforms were not specifically raised (Figure X). It is noteworthy that total changes in this enzyme attributable to the procedure could be accounted for by its appearance in the discard fluid: additionally there was no selective depletion of one or other component. Concurrently haptoglobin was shown to be below or at the lower limit of normal again consistent with a mild degree of erythrocyte breakdown (Figure XI).

FIGURE X

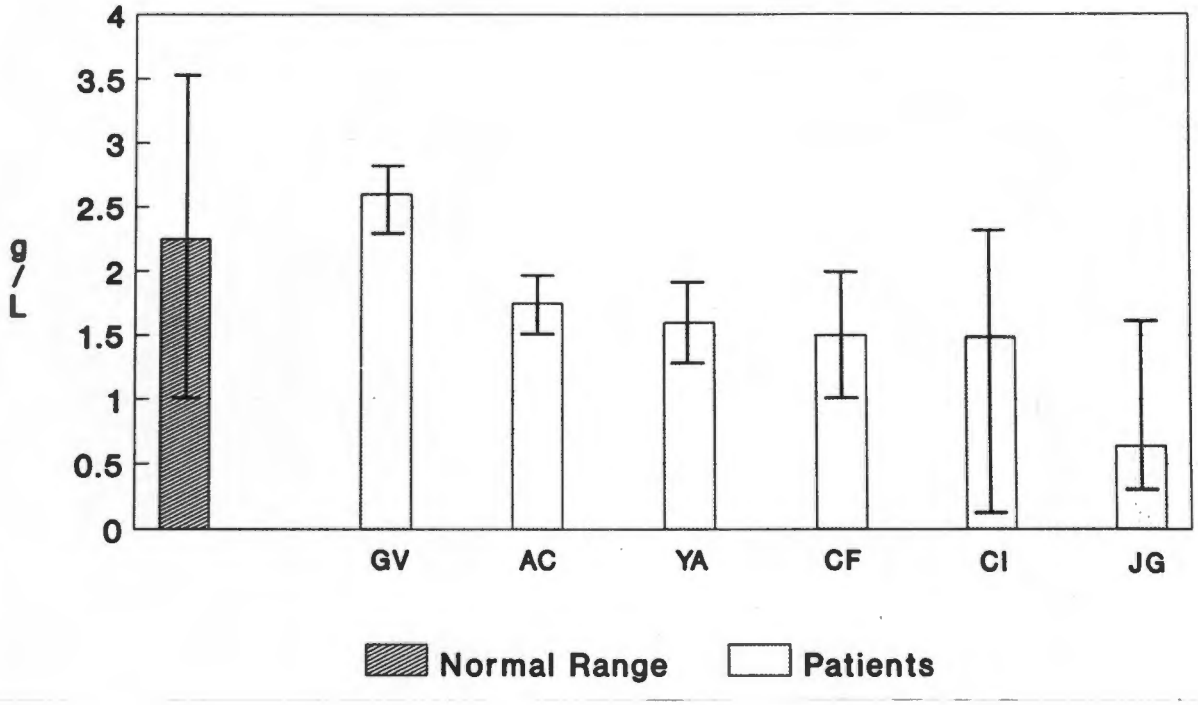
TOTAL LACTIC DEHYDROGENASE



A gradual rise in the pre-exchange value is seen over time. This is interpreted as being due to haemolysis but red cell isoform did not specifically increase. As far as is known the assay method did not change during the study period. In the presence of normal folate balance and a raised reticulocyte response there is no reason to ascribe these changes to ineffective erythropoiesis.

FIGURE XI

HAPTOGLOBIN



Appreciating that plasma exchange would acutely reduce haptoglobin levels, these were carried-out immediately before each procedure when steady state would have been re-established. In the six individuals there was, on average, marginal reduction which is consistent with ongoing, albeit low-level, shortening in red cell survival. Since post-exchange measurements were not carried-out comments about short-term rebound are not possible. The low level in JG, who had a porto-caval shunt, is noted but no further specific studies were undertaken to explore the impact on haptoglobin or red cell kinetics.

RADIONUCLIDE INVESTIGATIONS

It is noteworthy that, in homozygotes not yet on plasma exchange, mean red cell life-span was reduced. This establishes, for the first time, that haemolysis was associated with the disease itself.

Of particular note is that the impaired survival did not correlate with iron deficiency. Specifically ferritin levels were normal in the three patients prior to exchange and two of the three additionally had normal percentage saturation of transferrin. This observation supports the contention that the association with hypercholesterolaemia rather than iron deficiency, with or without anaemia, underlies the reduced mean red cell life-span. Interestingly, when the same data were analysed using different mathematical models, the curves were roughly similar but, in keeping with international practice, data are presented using the weighted-mean method (Table 6). In one patient where there was an opportunity to compare life-span before and after apheresis no further decrement was demonstrated.

In an alternative approach used to examine survival, loss of radiochromium in the urine gave equivocal results due to unreliable 24 hour collections (data therefore not shown).

ERYTHROPOIETIC RESPONSE

Reticulocytes

Compared to 64 controls the homozygous individuals had a consistently normal percentage. However, within this population, there was a marginal shift from low through medium to high fluorescent cells. As a result the maturation index was elevated which implies that subtly younger erythrocytes

TABLE 6

DEMOGRAPHIC DATA, IRON BALANCE AND RED CELL SURVIVAL

Patient Initials	Age (years)	Gender	% RDA	Iron Loss		$T_{50}Cr$ (days)	Mean Red Cell Lifespan (days)	% Saturation of Transferrin	Ferritin (µg/L)
				Discard Bag (mg)	In Urine (mg)				
GvdM	32	M	91	7	1.5	30.4	106.7	6	16
AC	24	F	47	4	2.05	24.2	73.7	16	15
YA	19	F	35	2.5	3.5	27.9	82.6	13	12
CF	19	M	152	8	3.4	31.0	111.8	14	7
CI	13	M	56	4	1.9	22.3	54.7	15	26
JG	23	F	35	2.5	0.18	ND	ND	ND	ND
TD	13	F	74	-	-	22.0	53.6	8	89
RI	25	F	ND	-	-	26.0	72.2	22	60
ES	24	F	ND	-	-	24.1	71.7	36	22
Referance Range			100	0	0.03	25 - 32	90 - 150	20 - 40	20 - 250

Iron intake shows reduced supply but absolute absorption, necessary for calculating balance, was not determined. Loss was measured by ICP-AES. In the discard plasma this is given for a two week period that includes a single representative apheresis. Urinary excretion for each exchange adds a further loss ranging from 0.18 to 3.5 mg. These calculations do not take into account additional minimum of 17 mg iron lost each month from diagnostic blood sampling.

The first six patients are those studied whilst standardised on the therapeutic plasmapheresis programme. Numbers 7, 8 and 9 are corresponding data from subjects not undergoing these procedures.

are being released by a marrow responding to the reduced red cell mass (Figure XII).

Collateral evidence for this statement, although weak, is found in the higher levels of pyruvate kinase and glucose-6-phosphate dehydrogenase when compared to physiological values (Figure XIII).

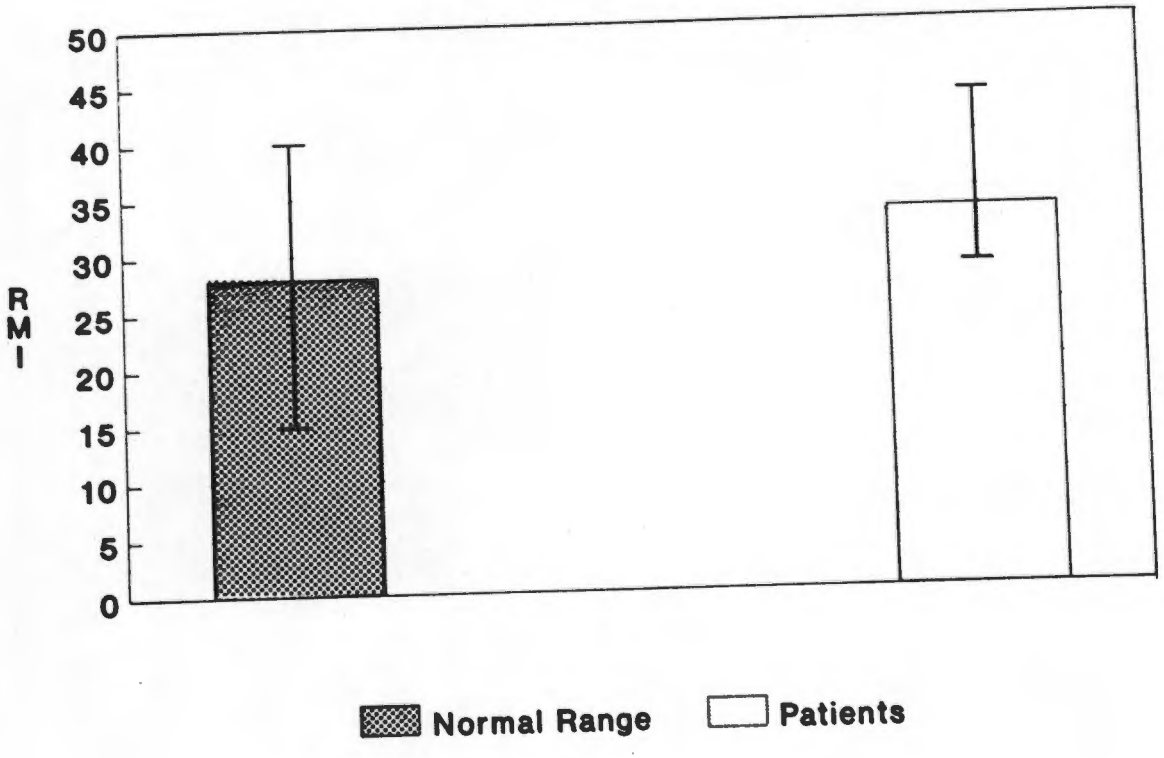
Additionally, in this context, was the plasma erythropoietin levels. In the first patient (AC) a blunted response was noted. However, with accumulation of further curves the observed to predicted (O/P) ratios were found to scatter widely about the mean normal value. This implies that enzyme output is appropriate and consistent with a intact functioning erythron (Figure XIV).

EKTACYTOMETRY

The need to examine integrity of the membrane was predicated on finding a shortened red cell survival *before* exchanges commenced and this was associated with an increased erythropoietin output which was nevertheless physiologically appropriate. Additionally this hormonal drive elicited the anticipated reticulocyte output in keeping with an intact response from the erythron. It is notable that, after repeated procedures, iron depletion occurred thereby raising the question of a causative mechanism. Arguing from previous precedent that stores are typically exhausted in paroxysmal nocturnal haemoglobinuria, where low-grade intravascular haemolysis results in haemosiderinuria, a similar defect was postulated. Accordingly intact erythrocytes and suitably prepared haemoglobin-free ghosts were studied.

FIGURE XII

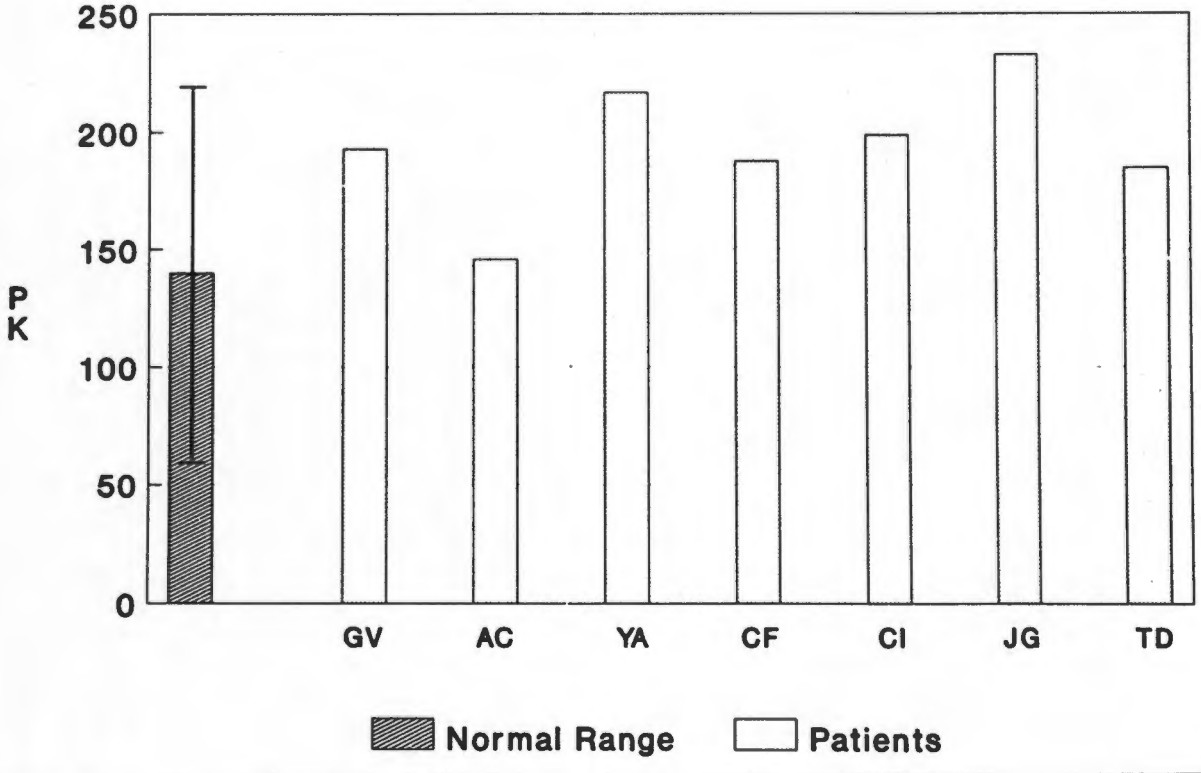
RETICULOCYTES

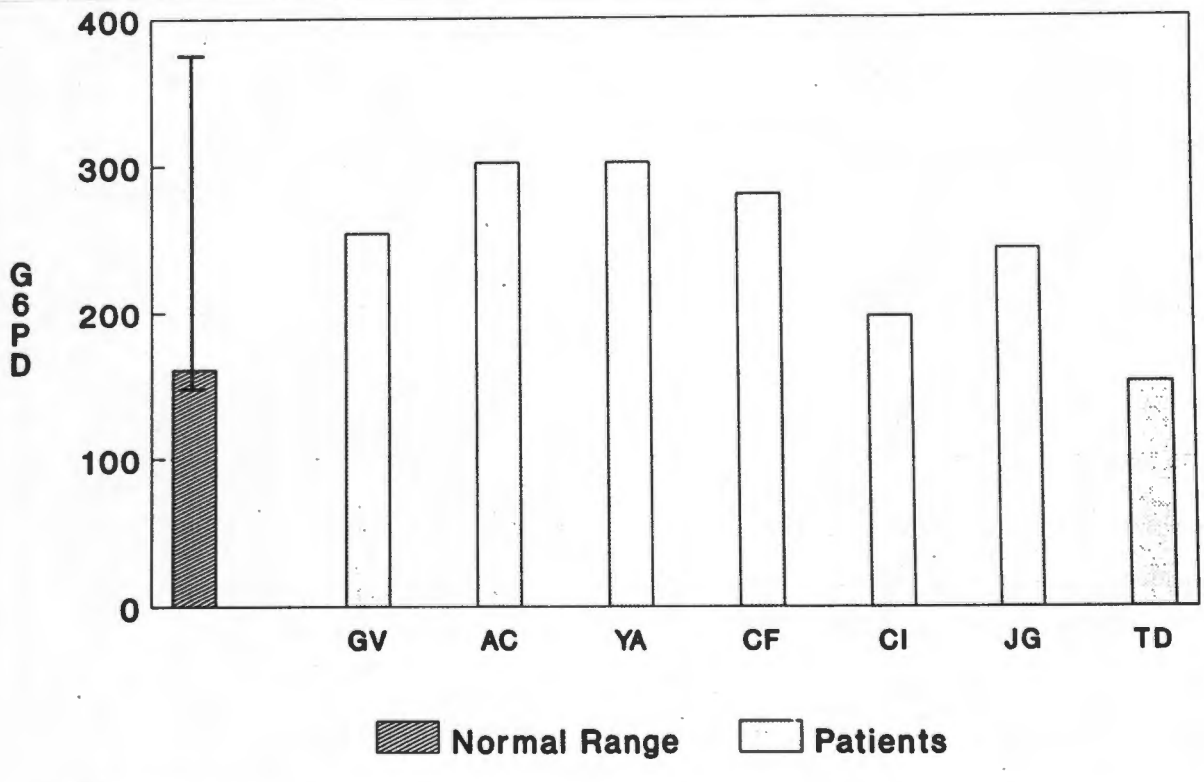


Although the total number are within the normal range the slightly elevated maturation index is consistent with a marginally younger, and therefore more brightly fluorescing population, being released by the stimulated bone marrow into the circulation.

FIGURE XIII

PYRUVATE KINASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE

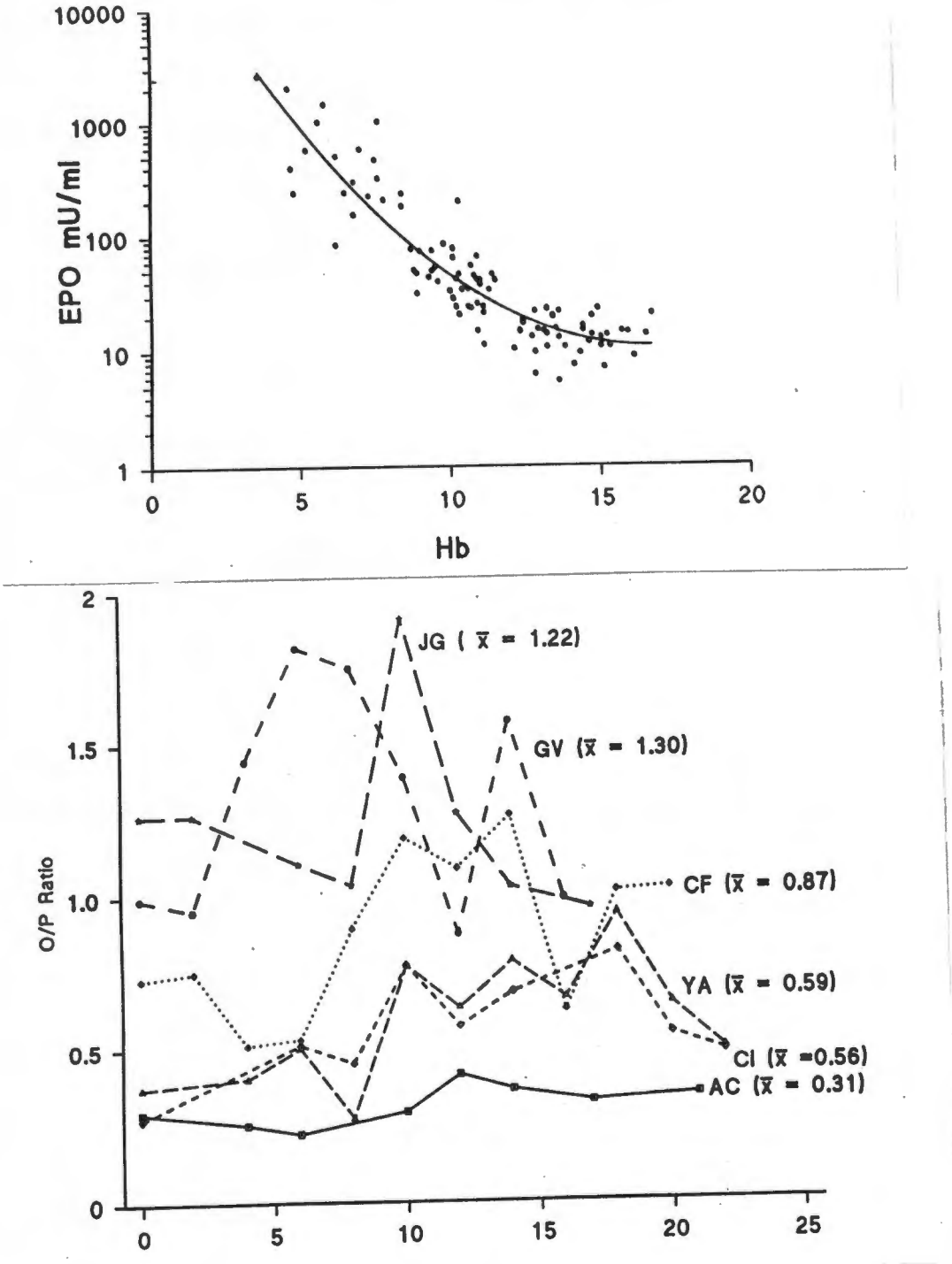




Both these red cell enzyme levels are in the upper normal range suggesting the presence of a younger cell population.

FIGURE X1V

PLASMA ERYTHROPOIETIN LEVELS



The top graph defines normal erythropoietin to haemoglobin ratio. The lower curves serially show over the two years of study (horizontal axis in weeks) the O/P ratio for the prevailing haemoglobin level. Although one patient (AC) has a blunted response the pattern for the total group scatters around the mean. This is consistent with an appropriate output for the degree of anaemia.

Each of the six patients on the plasma exchange programme were studied on at least five separate occasions and a normal control included in the run. In patients and controls the mean and standard deviation for whole cell deformability (n=42) was 123.6 (12.4) and 130.9 (15.1); the corresponding figures for deformability of isolated membranes (n=27) was 148.7 (30.6) and 153.21 (30.1); and for membrane fragmentation (n=27) was 84.1 (31.6) and 79.3 (31.6). These consistent trends fall just short of statistical significance.

In recognition of the fact that considerable machine variation occurs and that similar findings happen independently of the lipid abnormality but in the presence of iron deficiency oral supplementation was given over a six week period and all the individuals restudied on one single day. Fortuitously, due to variable compliance only some of the patients fully corrected their stores as reflected in plasma ferritin levels. Notwithstanding this the two groups again showed uniformly impaired whole-cell and membrane deformability and increased fragmentation of ghosts that was not affected by iron status. This observation suggests that the ektacytometric defect may be attributable to the hypercholesterolaemic state and not necessarily a function of the iron deficiency. It is acknowledged that the numbers and the differences are small. Furthermore, the confounding effect of a portacaval-shunt in two patients cannot be resolved on the available data: nevertheless, these findings are consistent with an intrinsically defective erythrocyte membrane associated with the elevated lipids (Table 7).

TABLE 7

EKTACYTOMETRY

IRON DEFICIENT		DEFORMABILITY				FRAGILITY	
		Whole Cell		Membrane		Membrane	
Name	Ferritin ug/L	Patient	Control	Patient	Control	Patient	Control
GV	14	81	85	102	115	57	56
YA	11	86	95	95	90	40	40
JG	9	75	86	99	104	46	37
ES	14	78	84	98	98	43	36
	Average Values:	**80**	**87.5**	**98.5**	**101.75**	**46.5**	**42.2**

IRON REPLETE		DEFORMABILITY				FRAGILITY	
		Whole Cell		Membrane		Membrane	
Name	Ferritin ug/L	Patient	Control	Patient	Control	Patient	Control
AC	36	76	77	91	110	37	33
CF	35	83	80	95	107	54	58
CI	41	92	87	98	96	56	42
PV	58	79	77	102	110	43	63
RI	36	85	94	108	106	51	38
	Average Values:	**83**	**83**	**98.4**	**105.8**	**48.2**	**46.8**

The lower limit of normal serum ferritin levels is 20ug/L. The crude data are presented as an osmoscan for whole cells and deformability and fragmentation scans respectively in the case of ghost membranes.[105,107]

IRON EXCRETION

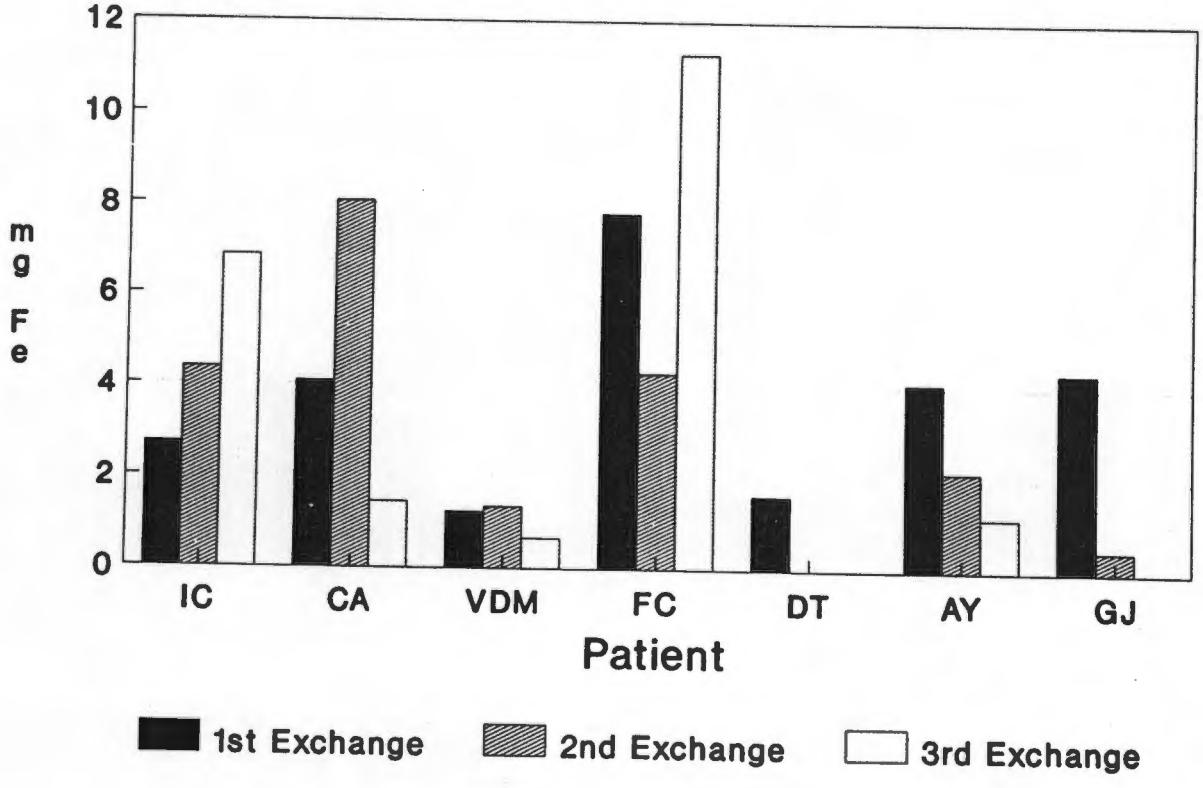
To link these observations it was necessary to demonstrate the route by which this element was being lost. Calorimetric techniques as used for serum determination fluctuated considerably when applied to the exchange fluid and urine so that the small amounts could not be reliably quantitated. Furthermore, ferritin could be demonstrated immunologically but its origin could not be attributed, with certainty, to red cells.

The only way out of the dilemma seemed to be direct measurement using inductively coupled plasma-atomic emission spectroscopy: this was then applied to examine exchange fluid and urine samples collected around the time of the procedure.

Two relevant facts emerged. Firstly, milligram quantities of iron were lost in each discard bag (Figure XV). Unfortunately it was not possible to identify the source or to correlate it with body stores prevailing at the time of the procedure. Secondly, a further significant excretory peak occurred in the post-apheresis urine. The latter seems to be related to the infusion of citrate used as anticoagulant that chelates the iron and this facilitates its excretion in the urine.

FIGURE XV

IRON LOSS IN DISCARD PLASMA



Inductively coupled plasma-atomic emission spectroscopy for the patients showed varying iron loss with each of three representative exchanges. This ranged from a high of 12 mg down to 1 mg.

CHAPTER VIII

SUPPLEMENTARY RESULTS

VISCOSITY

Plasma levels dropped dramatically after each run but had returned to normal within 14 days. Of note is a gradual elevation in whole blood value during the two years of study but this presently remains unexplained.

LEUCOCYTES

Neutrophils

There was a gradual upward trend in the count although the differential spread did not change. This remains unexplained since it was not associated with any consistent alteration in the procedure and no correlation was found with any of the other variables.

Function studies did not differ from controls and neither were influenced by apheresis.

Lymphocytes

Absolute numbers decreased as a result of the serial exchanges and this was demonstrably due to loss of the CD4 helper-T sub-set (Figure XVI).

Monocytes

Apart from a reduction after the individual runs there was rapid reconstitution and, in the longer term, no consistent effects.

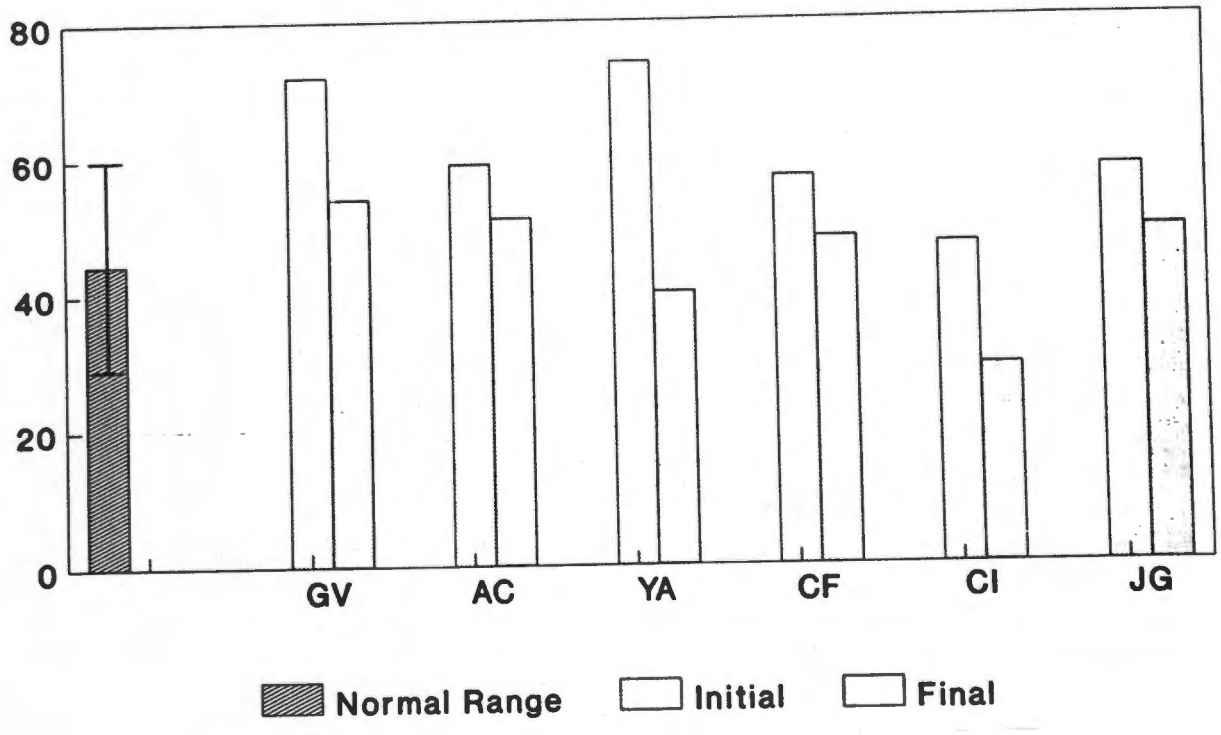
THROMBOCYTOPOIESIS AND PLATELET FUNCTION

Quantitative

There was a drop in count after each exchange which corrected itself (Figure XVII). However with repeated procedures mild thrombocytosis developed probably as a

FIGURE XVI

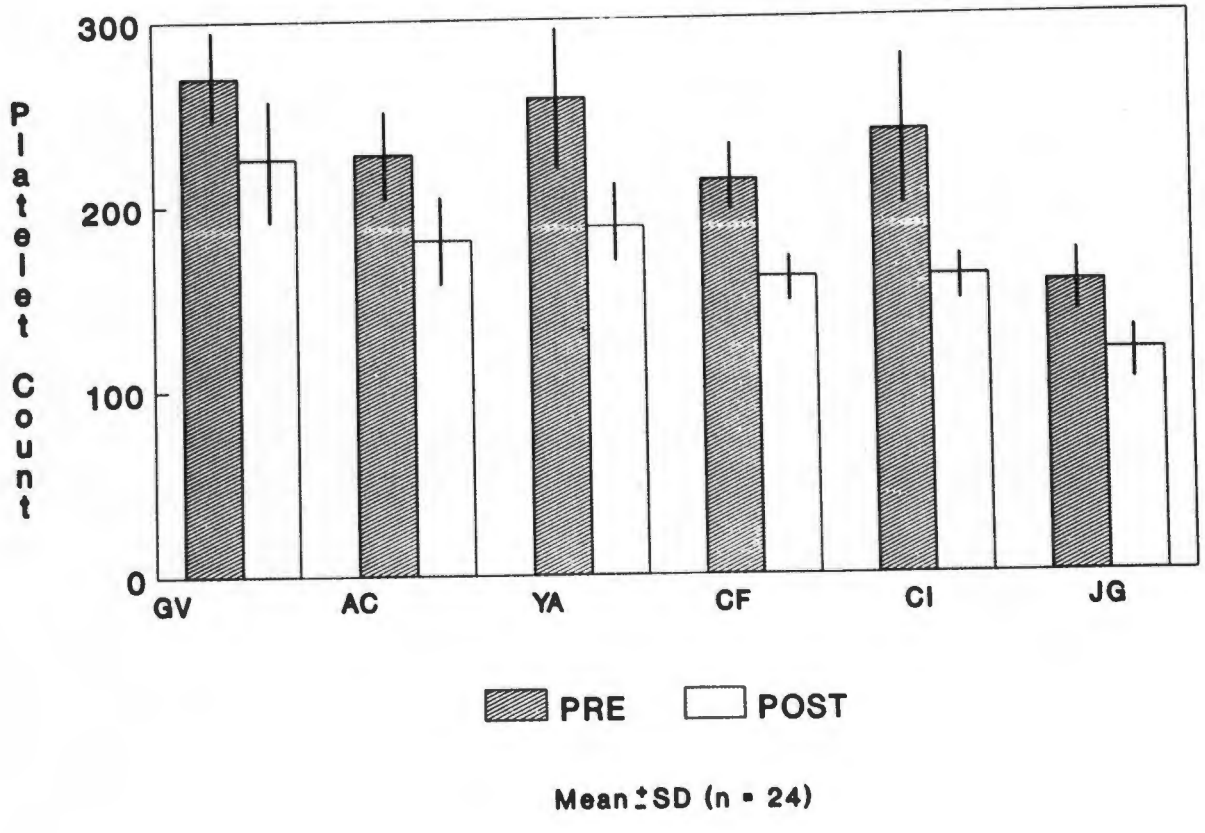
LYMPHOCYTE IMMUNOPHENOTYPING



The percentage (vertical axis) pan-T cell markers did not change but there was a reduction over the two year period in the CD 4 population. This is consistent with the previously reported observation arising in the closely allied procedure of plateletapheresis.[23]

FIGURE XVII

THE EFFECT OF PLASMA EXCHANGE ON THE PLATELET COUNT



The reduction consistently seen corrected within 48 hours. In no instance was this sustained or associated with thrombocytopenic bleeding. The vertical axis is platelet count $x10^9/L$.

reaction to the gradual development of iron deficiency that paralleled the fall in haemoglobin (Figure XVIII).

Qualitative

No difference in aggregometry could be attributed to plasmapheresis when patients were compared to controls using various agonists. Paradoxically, and appreciating the small numbers of studies as well as the variability and technique, it is notable that homozygous individuals, although starting off apparently less aggregatable than normals studied at the same time, have an increased *in vitro* response to these agents after completion of the plasma exchange (Table 8).

COAGULATION PROTEINS AND COMPLEMENT

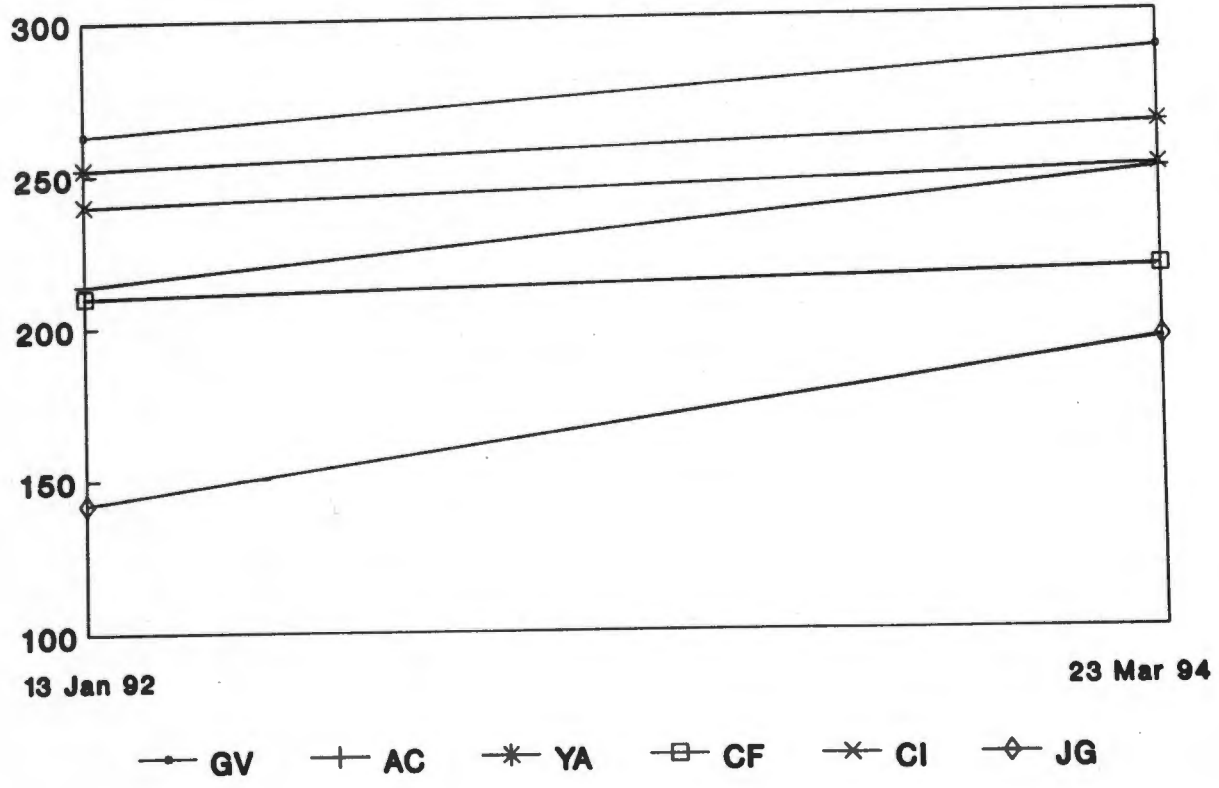
At the time of the apheresis there was a significant decrease in the proteins with prompt correction as previously reported.[1]

BIOCHEMISTRY

Measurements of urea, creatinine, electrolytes, calcium and liver enzymes were not affected. Ionizable calcium is known to fall but, because replacement was routinely given, it never dropped below the normal range.

FIGURE XVIII

DEVELOPMENT OF THROMBOCYTOSIS



With repeated procedures there was a mild increase in this measurement shown as platelet count 10^9/L on the vertical axis, that is attributable to the gradual development of iron deficiency.

TABLE 8

PLATELET AGGREGATION

	Pre	Post	Pre	Post
	Adenosine diphosphate 5 μm		**Ristocetin 1.5 μm**	
PATIENT	50(40-71)	50(40-80)	75(68-78)	78(56-84)
CONTROL	73(52-85)	69(50-84)	86(78-95)	93(78-95)
	Adenosine diphosphate 10 μm		**Adrenalin 10 μm**	
PATIENT	65(52-79)	56(46-68)	54(13-79)	19(10-79)
CONTROL	83(73-89)	81(79-89)	83(75-90)	83(76-90)
	Adenosine diphosphate 100 μm		**Collagen 15 μm**	
PATIENT	73(63-79)	76(65-79)	66(60-69)	66(38-78)
CONTROL	80(79-89)	77(71-89)	75(63-88)	73(63-88)

	ADP 5 μm	ADP 10 μm	ADP 100 μm	RISTO 1.5 μm	ADR 10 μm	COLL 15 μm
AC	+	+	+	-	O	+
CF	+	+	+	-	+	O
CI	+	+		+	+	+
JG	+	+	+	+	+	+
YA	+	+	-	-	+	-

In these studies the difference between pre- and post-exchange to a variety of agonists was compared (upper box). A plus sign shows that the difference for the patient was bigger than the difference for the control: a negative sign shows the opposite (lower box).

ADP = Adenosine diphosphate. COLL = Collagen.

ADR = Adrenalin. RISTO = Ristocetin.

CHAPTER IX

DISCUSSION

The cardiovascular ravages of severe hypercholesterolaemia are reflected in the high morbidity and mortality among Afrikaners of Sub-saharan Africa where a founder effect for these genes is clearly documented.[7-9] The clinical presentation of homozygous patients has been abundantly described with the disfiguring xanthomata, thickened tendons and intimal lipid deposits in the cerebral and coronary circulation causing stroke and premature myocardial-infarction with death at an early age.

Over the years attempts have been made to influence the relentless downhill course that these individuals follow. In heterozygotes dietary manipulation and lipid lowering agents are of some benefit but these turn out to be largely ineffective when profound low-density lipoprotein receptor dysfunction has been genetically transmitted as in the homozygote. Even extreme measures, including porto-caval anastomosis, have inconsistent and often little impact on survival.

Given the ominous outlook associated with this hyperlipidaemic phenotype it was to be anticipated that attempts to interfere with metabolism by use of HMG-CoA reductase inhibitors would be made, particularly in combination with a variety of apheresis procedures.[10,11]

While the different techniques for removing the offending cholesterol fractions continue to be intensively investigated, the ease and relatively low cost of centrifugal separation has

established it as the cornerstone for chronic lipid-depleting approaches. With careful attention to procedural detail, their safety over many thousands of apheresis on a world-wide basis has been extensively confirmed. The short-term complications are well documented. By way of contrast the anaemia that develops in the course of repetitive schedules has received scant attention. One or two reports have briefly noted its occurrence and then dismissed the finding as simply responding to iron but without apparently considering its pathogenesis or potential clinical implication.

It was for these reasons that a systematic investigation was undertaken to define incidence, characterise the haematologic lesion and then explore causation.

The initial step was to establish that, at presentation, the blood count was normal. Indeed this was universally the case and it was only after many plasmaphereses that a falling haemoglobin was noted. At this time the red cells were hypochromic and microcytic on examining the stained smear: both mean cell haemoglobin and volume were reduced when directly determined using modern particle counters.

Such acquired morphologic changes could best be explained by contraction and then absolute depletion of body iron stores. A number of mechanisms could theoretically be operating. In the first instance, dietary intake might be sub-optimal as a result of ingesting a diet low in fat and cholesterol. This postulate was confirmed when detailed questionnaires were completed by all the patients in the cohort showing that, in the majority, supply only marginally met the daily recommended allowance needed for normal

erythropoiesis. Given these circumstances maximal extraction from food would be necessary to meet physiologic needs. It is therefore notable that, in the absence of any evidence for loss from the gastrointestinal or genitourinary tract, compensation was sufficient to sustain a normal haemoglobin level with erythropoiesis being normochromic and normocytic. Thus, where data were available at patient registration adequate stores were present as reflected in iron, transferrin as well as its percentage saturation and serum ferritin levels.

This basal state was seen to be disrupted by repeated plasmaphereses leading to the development of anaemia. In seeking to explain the pathogenesis it was immediately appreciated that substantial amounts of iron were lost in consequence of repeated blood sampling. Of rather greater interest was the previously unreported possibility that red cell breakdown might occur in the extracorporeal circuit and lead to low-grade intravascular haemolysis: here precedent exists in patients with paroxysmal nocturnal haemoglobinuria. That such a mechanism might be operative in these subjects gained support from such a dramatic event associated with apheresis in a patient undergoing blood-group change *en route* to bone marrow transplantation where cyclophosphamide conditioning was believed to have damaged erythrocytes sufficiently for them to lyse during the procedure.[6]

To explore the possibility of procedure-related haemolysis participants were screened using serum lactic dehydrogenase and haptoglobin levels. The former showed a gradual rise over long periods of time consistent with an

evolving haemolytic process. However no particular isoform was selectively elevated so that the origin of the enzyme could not be precisely defined. Furthermore, it might reasonably have been anticipated that significant red cell breakdown would characterise the first few treatments where the circulating population included older cells. Accordingly, once these had been removed, the incoming reticulocytes should have been less vulnerable to such mechanical injury. Thus, in the face of normal folate values, the reason for the raised LDH remains unexplained. However it is speculated that the mild elevation of this enzyme may reflect iron depletion and impaired erythrocyte survival. Similarly plasma haptoglobin is at the lower limit of normal. This is again consistent with ongoing but low-level haemolysis. Such minimal depression may well reflect the relative resistance of younger cells circulating in the regularly exchanged individual.

Recognising that these assays did not provide specific information direct determination of mean red cell life-span, using radionuclides, was undertaken. Here the significant shortening found is worthy of particular comment since this defect was present in three subjects not undergoing plasma exchange and could therefore be ascribed to an intrinsically defective erythrocyte. It was scientifically interesting that, irrespective of whichever model is used for curve fitting, all gave comparable results and are within the recommendations made for this purpose by the International Committee for Standardisation in Haematology. Attempts to cross-check these findings were made by determining radiochromium excretion in the urine. These measurements did not give consistent figures,

explicable to a large extent by varying compliance with urine collection. The latter fact was evident when urinary creatinine levels were shown to be inappropriately low for body mass.

Advantage was taken of the isotope methods to measure blood loss related to the exchange. No significant leak of radio-labelled cells was found. Of further note is the observation that, where plasma volumes were determined before and after each procedure these, as well as blood volumes, were found to be unaffected. Stated differently the attention to detail insured that each was isovolaemic and that patients were left in neither positive nor negative fluid balance.

Taking all this data together it might reasonably be concluded that, prior to initiation of the apheresis protocol, a red cell defect already existed. Here slightly accelerated removal of the effete red cells by reticuloendothelial system was compensated for by simply accelerating erythropoiesis. In this context iron conservation is sufficient to maintain the otherwise precarious trace metal balance.

Any disturbance to this fragile homeostatic balance raises three other considerations.

Firstly, is the extent to which the erythron in these patients retains its capacity to respond to stress. That this is largely intact is supported by the finding of a compensated haemolytic state with a young erythroid population in the circulation as reflected in a reticulocyte maturation index consistent of their early release from the marrow compartment. Additionally, an enzyme pattern was found for glucose-6-phosphate dehydrogenase and pyruvate kinase associated with

such a stimulated response. Whether these levels were dampened by the concurrently present iron deficiency anaemia restricting haemoglobin synthesis is possible but a point not tested in this study. Conversely it is not inconceivable that the relatively modest shortening of survival and marginally reduced plasma haptoglobin are a consequence of somewhat greater resistance of young cells to breakdown by shear stresses found under these circumstances. It can be speculated that bigger differences may have been evident had it been possible to make these measurements when commencing aphereses rather than when the older cohort of red cells had already been depleted as a result of the initial exchanges.

Secondly and along the same lines, is the question as to why erythrocyte life-span is reduced particularly after sufficient time had elapsed for the older and more fragile members to have been removed. Again, with precedent from observations where lipid abnormalities give rise to impaired membrane integrity, the hypothesis was advanced that they were less deformable than normal. This would place them at continuing risk for damage during passage through the micro-circulation and particularly in the hostile environment of the spleen. Support for this concept was obtained using ektacytometry which is a method well suited to reproducing, in the laboratory, conditions that are thought to exist in the capillaries. The use of this technique showed a clear but non-significant trend for both this abnormality as well as an increased fragility. That statistical differences were not achieved may well reflect the small sample size that creates difficulty with obtaining a narrow standard deviation. It

would be proper to inject a note of caution into such an interpretation since further patients would need to be studied in order that this postulate can be more rigorously tested.

No discussion of these two functional defects would be complete without recognising that iron deficiency, in its own right, may give rise to similar findings. Indeed the entity of paradoxical hyperviscosity was described in just this context. That such nutritional disturbances may have contributed to the shortened survival remains a possibility but is thought to be excluded by repeating the ektacytometric measurements, following iron repletion, and demonstrating that they did not differ from serial studies carried-out in the same cases whilst they were on apheresis. An additional point in this context is that the population at greatest risk for *in vivo* and *in vitro* breakdown are the older red cells: however the compensatory situation prevailing in these patients should have diminished any influence of aged erythrocytes on deformability, fragility and life-span.

The third and final point was to uncover the link between commencement of aphereses, iron loss from the body leading to storage depletion and formation of intrinsically fragile cells. In the first instance repeated sampling during routine procedures, which increased when specific studies were being undertaken, placed a substantial strain on the quantity of this trace element available for haemoglobin synthesis. In looking further afield the limitations of suitable methods became apparent. Thus one appreciated that colorimetric techniques, as applied to plasma and urine, were relatively insensitive and consequently failed to demonstrate

haemosiderin. Similarly the unreliability of attempting to extrapolate excretion of this trace metal to the presence of immunologic determination of ferritin in the discard fluid provided a conundrum. Was the element getting-out but in a form that escaped detection by these cruder methods? Using ICP-AES it was possible to unequivocally demonstrate that this was the case and, furthermore, this loss became significant when the marginal intake was considered. Thus milligram quantities were revealed to be present in the exchange fluid discarded with each run. In addition, there were peaks in the urinary iron concentration immediately following each exchange. This could best be explained by chelation of the free metal from the circulation in response to citrate used as the anticoagulant in the routine procedures.

Under these circumstances the reduced amounts available from the diet are such that negative balance is present in some cases. In others any minor additional loss from normal menstruation or repeated blood sampling carried-out during the course of their management is probably sufficient to tip the scales in this adverse direction.

CHAPTER X

SUMMARY AND CONCLUSIONS

Patients with homozygous familial hypercholesterolaemia on long-term plasmapheresis programmes have occasionally been noted to have an iron-responsive anaemia. To date no attempt has apparently been made to define its incidence or explore pathophysiology. Accordingly this thesis reports the universal occurrence of a falling haemoglobin level in all these individuals and its systematic study, in four consecutive phases, over a two year period.

The first of these documented that, wherever data was available at registration, haematologic and iron values were normal but, in the course of serial plasmapheresis, there is the predictable development of hypochromic and microcytic erythropoiesis.

In the second, after iron loss from gastrointestinal and genitourinary tract had been excluded, availability of this haematinic from the low fat diet routinely ingested was examined by questionnaire and found only marginally adequate to meet minimal daily requirements for normals and therefore likely to be insufficient should demands increase for any reason. One such drain on available stores would be loss through repeated blood sampling needed to ensure safety of the procedures and this might well become more acute at times when research protocols necessitated additional collections. A further mechanism might be the occurrence of haemosiderinuria due to low-grade intravascular haemolysis as occurs so distinctively in paroxysmal nocturnal haemoglobinuria.

Suggestive evidence for this type of pathway to be operative was found in the minimally reduced haptoglobin and raised serum lactic dehydrogenase levels. Haematologically this postulate was supported by the presence of a young red cell population reflected in a subtly raised reticulocyte maturation index, coupled with glucose-6-phosphate dehydrogenase and pyruvate kinase levels at the upper limit of normal. Shortened erythrocyte survival was proven to be present, using radiochromium red cell labelling prior to either creation of an arteriovenous fistula or commencing the apheresis protocol. Such a lesion has not previously been described. At the same time erythropoietin levels were appropriate for the degree of anaemia supporting an intact bone marrow response. These observations are consistent with there being an enhancement of the normal extravascular route for removal of effete cells by the reticuloendothelial system which is, in effect, an iron-conserving process.

A third phase was designed to explore the impact of plasma exchange on disturbing this fragile homeostatic balance arguing that the cells may be intrinsically abnormal as a consequence of the hypercholesterolaemic environment in which they circulate. Ektacytometry was employed to demonstrate reduced deformability of the intact cells: this presumably resided in the membrane since the lesion persisted when erythroid ghosts were studied. Additionally isolated membranes showed increased fragility. This combination of the two defects might reasonably be interpreted as predisposing erythrocytes to accelerated mechanical breakdown either in the extracorporeal circuit or, once returned to the body, within

the micro-circulation perhaps particularly in the spleen. Such mechanisms, active over long periods of time, could aggravate the negative iron balance.

In the fourth and final series of experiments the loss of this trace metal to the external environment was explored. Standard colorimetric methods gave equivocal results probably due to their lack of sensitivity. Ferritin could be recovered from the exchange fluid but methodology was not available to determine its origin. Conversely the use of ICP-AES showed milligram losses in the discard fluid with each exchange and, concurrently, peaks of urinary excretion at the time of each procedure. This additional drainage on already compromised stores could not be compensated for by the sub-optimal intake from the diet so that an impaired delivery to plasma became rate limiting for red cell formation. Relevantly iron deficiency, in its own right, was excluded as the dominant cause for the ektacytometrically demonstrated lesions and, presumably therefore, the shortened erythrocyte survival.

It is concluded, from the data presented, that the development of hypochromic and microcytic anaemia evolves as a consequence of serial plasmaphereses in these individuals and does so through a number of inter-related factors. One is inadequate iron intake. A second is blood loss through repeated sample collections. Thirdly, and perhaps scientifically the most interesting, is superimposition of loss in the exchange fluid at the time of plasmapheresis when intrinsically fragile red cells undergo accelerated breakdown.

Two final comments are appropriate.

Firstly this appears to be the initial report of such a haematologic defect in the homozygous patients. Secondly, the study has direct clinical relevance. Thus the simple expedient of supplementation will prevent symptoms attributable both to falling haemoglobin and any superimposed paradoxical hyperviscosity resulting from iron deficiency.

CHAPTER XI

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